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(54) Title: FLAVIVIRUS NS1 SUBUNIT VACCINE

(57) Abstract: The present invention relates to NS1 proteins or parts thereof of Flaviviruses, in particular of Dengue viruses useful for vaccination against said Flavivirus and against one or more other Flaviviruses. The invention further concerns the NS1 protein or parts thereof of one Dengue virus serotype, in particular serotype 2, useful for vaccination against Dengue viruses from all serotypes. The invention further concerns DNA comprising an expression cassette coding for a Flavivirus NS1 or parts thereof, vectors, comprising said DNA and vaccines containing or expressing a Flavivirus NS1.

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## Flavivirus NS1 Subunit Vaccine

The present invention relates to NS1 proteins or parts thereof of Flaviviruses, in particular of Dengue viruses useful for vaccination against said Flavivirus and against one or more other Flaviviruses. The invention further concerns the NS1 protein or parts thereof of one Dengue virus serotype, in particular serotype 2, useful for vaccination against Dengue viruses from all serotypes. The invention further concerns DNA comprising an expression cassette coding for a Flavivirus NS1 or parts thereof, vectors comprising said DNA and vaccines containing or expressing a Flavivirus NS1.

## Background of the invention

The etiological agent of the dengue fever is the Dengue virus, belonging to the Flavivirus genus of the family Flaviviridae (Burke and Monath, 2001). A particularly important subgroup of Flaviviruses is the group of so called mosquito-borne Flaviviruses, i.e. Flaviviruses that are transmitted by mosquitos. This group comprises in addition to the above mentioned Denguevirus other important viruses such as the West nile virus, the Japanese encephalitis virus and the Yellow fever virus (Fields Virology, ed. by Fields B.N., Lippincott-Raven Publishers, 3<sup>rd</sup> edition 1996, ISBN: 0-7817-0253-4, pages 931-1034). Typical diseases transmitted by these viruses are West nile fever and West nile encephalitis induced by the West nile virus, encephalitis induced by the Japanese encephalitis virus, Yellow fever induced by the Yellow fever virus and Dengue fever, dengue hemorrhagic fever (DHF; see below) and Dengue shock syndrome (DSS) induced by the Dengue virus.

Flaviviruses are enveloped, single-stranded, positive-sense RNA viruses formed by three structural proteins: the capsid protein (C) that

forms a nucleocapsid in association with the viral genome, which is surrounded by a lipid bilayer in which are anchored the M (membrane) and E (envelope) proteins. The genome is approximately 11kb long and contains a single open reading frame encoding a polyprotein precursor of  
5 about 3400 amino acid residues. Individual viral proteins are generated from this precursor by the action of cellular and viral proteases. The three structural proteins (C, M and E) are derived from the N-terminal part of the polyprotein and are followed by seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Lindenbach and Rice, 2001).

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Glycoprotein NS1, present in all Flaviviruses, appears to be essential for virus viability. Dengue virus NS1 is secreted from mammalian infected cells in a soluble hexameric form (Flamand et al., 1999). This noncovalently bound hexameric complex is formed by 3  
15 dimeric subunits and has a molecular mass of 310 kDa. Dimerization is a prerequisite for NS1 protein export to the plasma membrane, where it remains as the unique viral resident protein of the infected cell surface.

In mammalian cells, but not in insect cell lines that support dengue  
20 infection, part of the transported NS1 is released into the extracellular milieu. Extracellular NS1 is secreted either as a soluble protein, which exist in a higher hexameric oligomeric form, or in association with microparticles but not with virions. In addition, NS1 has been found circulating in sera from dengue virus infected patients, suggesting that  
25 secretion of NS1 may be an important event in Flavivirus infection in the human host. During the course of a Flavivirus infection, the NS1 protein evokes a strong antibody response, which helps to clear the infecting virus from the host, presumably through a complement-mediated pathway (Schlesinger, J.J. et al., 1987) and antibody-dependant cell cytotoxicity  
30 (ADCC) (Schlesinger, J.J. et al., 1993).

The Dengue virus, with its four serotypes Dengue virus serotype 1 (Den-1) to Dengue virus serotype 4 (Den-4), is the most important member of the Flavivirus genus with respect to infections of humans and produces diseases that range from flu-like symptoms to severe or fatal illness, dengue haemorrhagic fever with shock syndrome. Dengue outbreaks continue to be a major public health problem in densely populated areas of the tropical and subtropical regions, where mosquito vectors are abundant.

10           The concern over the spread of dengue infection and other diseases induced by mosquito-borne Flaviviruses in many parts of the world has resulted in more efforts being made towards the development of dengue vaccines, which could prevent both dengue fever (DF), and dengue hemorrhagic fever (DHF) and in vaccines useful to protect the vaccinated individual against infections induced by some or all mosquito-borne Flaviviruses.

20           While most cases of DF are manifested after the first infection by any of the four serotypes, a large percentage of DHF cases occur in subjects who are infected for the second time by a serotype which is different from the first infecting serotype of dengue virus. These observations give rise to the hypothesis that sequential infection of an individual with antibody against one dengue serotype by a different virus serotype at an appropriate interval may result in DHF in a certain number of cases. Antibody-dependant enhancement (ADE) has been demonstrated *in vitro* for dengue viruses, as well as other enveloped viruses, and is considered to be an important mechanism in the pathogenesis of DHF.

30           It has also been observed that DHF usually emerges in geographic areas where multiple (three or four) virus serotypes co-circulate. In

regions with endemic DHF such as Southeast Asian countries, the age-specific attack rate is higher in children, and the number of DHF cases decreases in higher age groups. This roughly corresponds with the increasing seroprevalence to dengue, indicating that natural infection may evoke protective immunity. This phenomenon is not unlike that observed with other viral infections such as hepatitis A virus. Anecdotal clinical observations have shown that patients may experience DHF twice (Nimmannitya *et al.*, 1990) but this is rare, and it is difficult to identify accurately the serotypes causing the second and subsequent infections. So far, there has been no reports of a forth infection in the same individual, despite the fact that all four dengue virus serotypes circulate in the same area. This suggests that, in nature, infection by two or three dengue virus serotypes in the same individual may result in cross-reactive antibodies or even a cross-reactive cytotoxic lymphocyte response. This may modulate or protect against infection by the remaining dengue virus serotypes in nature.

At present there are no approved dengue vaccines. Today, prevention of dengue virus infection is dependent upon control of the principal mosquito vector, *Aedes aegypti*. Insecticide resistance, lack of technical and financial support that would enable local health departments to maintain effective mosquito control programs, and continuing geographic spread of both the vector mosquitoes and dengue viruses make it practically impossible to prevent dengue infections by current mosquito control programs. Therefore, development of safe and effective vaccines against all four serotypes of dengue virus has been designated by the WHO as a priority for the most cost-effective means to prevent dengue virus infection. The WHO has recommended that the ideal vaccine against dengue and DHF should be the kind that can prevent infection caused by all serotypes so that sequential infection cannot happen.

To this end WO 98/13500 proposes to use a recombinant Modified Vaccinia Virus Ankara (MVA) expressing antigens from all Dengue virus serotypes or to use four recombinant MVA wherein each of the  
5 recombinant MVA expresses at least one antigen of one Dengue virus serotype. Both strategies provide very promising strategies to vaccinate against all Dengue virus serotypes. However, it is desirable to provide a single subunit vaccine that upon administration results in an immune response against more than one Flavivirus or against more than one  
10 serotype of Dengue virus, preferably against all Dengue virus serotypes. Moreover, WO 98/13500 discloses a recombinant MVA encoding Dengue virus NS1. WO 98/13500 does not disclose that an antigen derived from one Dengue virus serotype elicits an immune response not only against the Dengue virus serotype from which the antigen is derived but also  
15 against antigens derived from other Dengue virus serotypes.

WO 99/15692 discloses a recombinant MVA containing and capable of expressing one or more DNA sequences encoding Dengue virus antigens not able to effect immune enhancement or antibody dependant  
20 enhancement. WO 99/15692 does not disclose that an antigen derived from one Dengue virus serotype elicits an immune response not only against the Dengue virus serotype from which the antigen is derived but also against antigens derived from other Dengue virus serotypes.

25

### **Object of the invention**

Thus, it is an object of the invention to provide a vaccine derived from a Flavivirus or a Flavivirus serotype that is stable, can easily be produced and that leads to an immune response that protects the vaccinated  
30 individual not only against the Flavivirus or the Flavivirus serotype from which the vaccine is derived but also against other Flaviviruses or

Flavivirus serotypes. It is a particular object of the present invention to provide a vaccine derived from a mosquito-borne Flavivirus that protects the vaccinated individual not only against the mosquito-borne Flavivirus or Flavivirus serotype from which the vaccine is derived but also against  
5 other mosquito-borne Flaviviruses or Flavivirus serotypes. It is a further object to provide a vaccine that is derived from one Dengue virus serotype and that protects an individual against an infection with at least two, preferably all Dengue virus serotypes.

10

### **Detailed description of the invention**

These objects have been solved using the NS1 protein or parts thereof of a Flavivirus and DNA sequences comprising an expression cassette coding for a Flavivirus NS1 protein or a part thereof, respectively. In particular,  
15 the object to provide a vaccine that is derived from one mosquito-borne Flavivirus and that protects an individual against an infection with the mosquito-borne Flavivirus from which the vaccine is derived but also against an infection with at least one other mosquito-borne Flaviviruses has been solved by using the NS1 protein or a part thereof of a mosquito-  
20 borne Flavivirus, in particular the Dengue virus, preferably Dengue virus serotype 2 and corresponding DNA sequences, respectively. More specifically the object to provide a vaccine that is derived from one Dengue virus serotype and that protects an individual at least against an infection with at least two, preferably at least three, more preferably all  
25 Dengue virus serotypes and preferably also against the infection with other Flaviviruses, in particular mosquito-borne Flaviviruses such as the Japanese encephalitis virus, the Yellow fever virus and West Nile virus has been solved by using the NS1 protein or a part thereof of a Dengue virus, in particular of Dengue virus serotype 2 and corresponding DNA  
30 sequences, respectively.

As it is shown in more detail in the experiment section the NS1 protein derived from a Dengue virus of one serotype expressed *de novo* after vaccination can evoke an antibody response that will cross react with NS1 proteins of Dengue virus serotype 1, 2, 3 and 4 plus NS1 from other members of the Flavivirus genus such as Japanese encephalitis virus, Yellow fever virus and West Nile virus. Thus, NS1 protein from one Dengue virus serotype origin is a universal DHF subunit vaccine for simultaneous protection against at least two, more preferably three, even more preferably all four serotypes of dengue virus and further against one or more other viruses of the genus Flavivirus. Since in this subunit vaccine strategy no E protein is involved, there should be no risk of Antibody Dependant Enhancement (ADE) upon subsequent exposure to any of the serotypes of dengue and therefore no vaccine related DHF should be induced during natural outbreaks of dengue infection.

15

The NS1 protein may be expressed from a nucleic acid, preferably a DNA comprising an expression cassette coding for at least a Flavivirus NS1 protein or a part thereof. The term "at least" in this context is to be interpreted in that the expression cassette may further encode additional proteins/peptides, either as separate proteins/peptides or fused to the NS1 protein or part thereof as defined in more detail below. In the context of the present invention the term „DNA“ refers to any type of DNA, such a single stranded DNA, double stranded DNA, linear or circular DNA or DNA in the form of a plasmid or a viral genome. Since Flaviviruses are RNA viruses the DNA coding for the Flavivirus NS1 protein is a non-naturally occurring DNA, such as a cDNA or a synthetic DNA.

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The term „expression cassette coding for a Flavivirus NS1 protein or part thereof“ is to be interpreted in that the coding sequence of a Flavivirus NS1 protein or a part thereof is preceded by elements controlling the transcription, in particular the initiation of transcription.

30



Examples for such transcriptional regulatory elements are prokaryotic promoters and eukaryotic promoter/enhancers. Preferred eukaryotic promoter/enhancers are the human Cytomegalovirus immediate early promoter/enhancer and poxvirus promoters such as the 7.5 promoter and  
5 the poxvirus minimal promoter as disclosed in the example section. The sequence of the poxvirus minimal promoter is shown in Figure 2 as well as in SEQ:ID No. 9. The expression cassette may further contain elements controlling the termination of transcription such as prokaryotic termination elements or eukaryotic poly A signal sequences, if necessary.

10

The expression cassette may express only the NS1 protein or part thereof of a Flavivirus or may express the NS1 protein or part thereof together with one or more further Flavivirus proteins/peptides, wherein the NS1 protein or part thereof and the further proteins/peptides are  
15 produced as separate proteins/peptides or as fusion proteins/peptides. If not defined otherwise in this description the term „peptide“ in the context of the present invention refers to a contiguous amino acid sequence stretch of at least 10 amino acids, more preferably of at least 20 amino acids, most preferably of at least 25 amino acids.

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The further Flavivirus protein is not the entire E-protein since this protein seems to be involved in the development of DHF. Thus, if the further Flavivirus peptide is derived from the E-protein it should comprise less than 40 amino acids, preferably less than 35 amino acids. If amino  
25 acid sequence stretches derived from the E-protein are expressed together with the NS1 protein or part thereof it should have been verified that this amino acid stretch does not comprise an epitope that is involved in the generation of ADE and DHF.

30 If the expression cassette expresses in addition to the NS1 protein or part thereof a further Flavivirus protein/peptide as separate

proteins/peptides the expression cassette may comprise an Internal ribosome entry site (IRES) between the sequence encoding the NS1 protein or part thereof and the sequence encoding the further Flavivirus protein. IRES elements are known to the person skilled in the art.

- 5 Examples for IRES elements are the picornaviral IRES elements or the 5'non-coding region of the hepatitis C virus.

Alternatively the nucleotide sequence encoding the NS1 protein or part thereof may be fused to a DNA sequence encoding further Flavivirus proteins/peptides in such a way that a fusion protein between the NS1  
10 protein or part thereof and the further Flavivirus protein/peptide is produced. If the NS1 protein or part thereof and the further Flavivirus protein/peptide are to be produced as fusion proteins/peptides the respective coding sequences are fused in frame.

15

In a preferred embodiment the DNA sequence encoding the NS1 protein or part thereof is preceded by the sequence encoding the glycosylation signal sequence of the E-protein. According to this embodiment a fusion protein is produced that comprises the E-protein  
20 glycosylation signal sequence fused to the NS1 protein or part thereof. As pointed out above the E-protein derived amino acid stretch should be as short as possible and it should be excluded that this amino acid stretch contains an epitope involved in the generation of ADE and DHF. The glycosylation signal sequence of the E-protein fulfils these requirements.

25

In an alternative preferred embodiment the expression cassette used according to the present invention contains as only Flavivirus sequence the sequence encoding the NS1 protein or part thereof. Thus, in this preferred embodiment the expression cassette according to the  
30 present invention does not express any other peptides/proteins from

other parts of the Flavivirus genome, in particular not the NS2A or the E protein.

In a further alternative embodiment the DNA according to the present invention expresses the NS1 protein or part thereof as a fusion protein with proteins/peptides that are not derived from a flavivirus. Such proteins/peptides comprise non-flaviviral signal sequences or sequences that are useful for the detection or purification of the expressed fusion protein, such as tags.

To understand the general structure of the Flavivirus sequence in the expression cassette used according to a preferred embodiment of the present invention it is helpful to summarize briefly the genome structure of Flaviviruses: During a natural Flavivirus infection the virus produces a single polyprotein which is then cleaved first by host cell proteases and then virus encoded proteases into the following proteins: C, PrM and M, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (protein order along the polyprotein precursor). Therefore, a DNA sequence, in particular a cDNA sequence coding for the NS1 protein or part thereof must require the addition of a "ATG" start codon. In a preferred embodiment the start ATG is then followed by a sequence encoding a glycosylation signal so that the newly synthesized NS1 protein becomes glycosylated in the endoplasmic reticulum. Such signal sequences are known to the person skilled in the art. Finally, the protein-coding cassette needs a stop codon, which might be a TAG added to the 3' terminal end of the protein coding cDNA sequence. In the example used in this invention the "ATG+signal sequence" element was derived from the sequence encoding the hydrophobic C-terminal end of the E protein (the last 28 amino acids, which for the Dengue virus New Guinea strain („NGC strain“, GeneBank accession number AF038403) starts with the amino acid M (ATG). A

typical expression cassette according to the present invention is shown in Figure 2 and as SEQ:ID No 9 and SEQ:ID No. 10.

Thus, in summary this embodiment concerns the use of a DNA  
5 comprising an expression cassette comprising the sequences coding for a  
Flavivirus NS1 protein or part thereof, wherein the coding sequence is  
preceded by a start codon ("ATG") and a sequence encoding a signal  
sequence for glycosylation, preferably derived from the E-protein as  
defined above and wherein the coding sequence is terminated by a stop  
10 codon of translation (Figures 1A, 1C and 2, SEQ:ID 5-10).

The DNA sequence that may be used according to the present  
invention encodes a Flavivirus NS1 protein or part thereof. The term  
"Flavivirus" refers to any Flavivirus. More preferably the term "Flavivirus"  
15 refers to mosquito-borne Flaviviruses such as the West Nile virus, the  
Japanese encephalitis virus, the Yellow fever virus and the Dengue virus.  
The NS1 protein or part thereof derived from one mosquito-borne virus  
encoded by a DNA according to the present invention should protect the  
vaccinated individual not only against an infection with the virus or virus  
20 serotype from which the vaccine is derived but also against the infection  
with other mosquito-borne viruses or other serotypes of the virus from  
which the vaccine is derived. The NS1 protein can preferably be of any  
Dengue virus serotype. More preferably the NS1 protein coding sequence  
is derived from a Dengue virus serotype 2 such as the Dengue virus New  
25 Guinea strain („NGC strain“, GeneBank accession number AF038403).  
The terms "subtype" and "serotype" are used interchangeably throughout  
this description.

The term „part thereof“ in the context of the term „NS1 protein or  
30 part thereof“ refers to an amino acid stretch of the NS1 protein, which is  
sufficiently long to induce a specific immune response against the NS1

protein from which the „part thereof“ is derived. If the Flavivirus is a Dengue virus the amino acid stretch should be an amino acid stretch that provokes an immune response in a vaccinated animal including a human against the NS1 proteins of all Dengue virus serotypes. In the examples  
5 section it is shown how the person skilled in the art can determine whether an NS1 protein or part thereof induces an immune response specific for all Dengue virus serotypes. According to a preferred embodiment the Flavivirus DNA sequence encodes the entire NS1 protein. Thus, the term “NS1 protein or part thereof” relates to the entire  
10 sequence of naturally occurring NS1 proteins and shorter epitope stretches that still elicit an immune response.

Moreover the term “NS1 protein” also relates to derivatives of naturally occurring NS1 proteins. Such a derivative may be a protein that  
15 has one or more amino acid substitutions, deletions and/or insertion with respect to the naturally occurring NS1 protein. By way of example such a derivative is a protein that has a homology in the amino acid sequence of at least 50%, preferably of at least 75%, more preferably of at least 90%. Consequently, the term “part thereof” also relates to parts of such a NS1  
20 protein derivative.

In summary one of the most preferred embodiments of the present invention is to use a DNA comprising an expression cassette coding for a mosquito-borne Flavivirus NS1 or part thereof, wherein the Flavivirus is  
25 preferably the Dengue virus, in particular Dengue virus serotype 2, and wherein the expression of the NS1 protein or part thereof is controlled by a transcriptional regulatory element. More preferably the DNA according to the present invention encodes the NS1 protein or part thereof as a fusion protein with a glycosylation signal sequence.

The invention further refers to vectors comprising a DNA as described above and to the use of said vectors to induce an immune response according to the present invention. The term „vector“ refers to any vectors known to the person skilled in the art. A vector can be a  
5 plasmid vector such as pBR322 or a vector of the pUC series. More preferably the vector is a virus vector. In the context of the present invention the term „viral vector“ or “virus vector” refers to an infectious virus comprising a viral genome. In this case the DNA of the present invention is to be cloned into the viral genome of the respective viral  
10 vector. The recombinant viral genome is then packaged and the thus obtained recombinant vectors can be used for the infection of cells and cell lines, in particular for the infection of living animals including humans. Typical virus vectors that may be used according to the present invention are adenoviral vectors, retroviral vectors or vectors on the basis  
15 of the adeno associated virus 2 (AAV2). Most preferred are poxviral vectors. The poxvirus may be preferably a canarypox virus, a fowlpoxvirus or a vaccinia virus. More preferred is modified vaccinia virus Ankara (MVA) (Sutter, G. *et al.* [1994], Vaccine 12: 1032-40). A typical MVA strain is MVA 575 that has been deposited at the European Collection of Animal  
20 Cell Cultures under the deposition number ECACC V00120707. Most preferred is MVA-BN or a derivative thereof which has been described in the PCT application WO 02/42480 (PCT/EP01/13628). The content of this application is included in the present application by reference. MVA-BN has been deposited at the European Collection of Animal Cell Cultures  
25 with the deposition number ECACC V00083008. By using MVA-BN or a derivative thereof the additional technical problem has been solved to provide a particular safe virus vaccine against Flaviviruses since it has been shown that the MVA-BN virus vector is an extremely attenuated virus. In particular, it has been demonstrated that MVA-BN is more  
30 attenuated than the MVA strains known before in the prior art. MVA-BN is derived from Modified Vaccinia Ankara virus and is characterized by the

loss of its capability to reproductively replicate in human cell lines. MVA-BN is safer than any other known vaccinia virus strains due to a lack of replication in humans. In the preferred embodiment the invention concerns as a viral vector containing the DNA as defined above MVA-BN and derivatives of MVA-BN. The features of MVA-BN, the description of biological assays allowing to evaluate whether a MVA strain is MVA-BN or a derivative thereof and methods allowing to obtain MVA-BN or a derivative thereof are disclosed in WO 02/42480.

10           The term "derivatives" of the virus as deposited under ECACC V00083008, i.e. derivatives of MVA-BN, is used in the present application as defined in WO 02/42480. In the following the features of a derivative of MVA-BN are shortly summarized. For more detailed information regarding the definition of a derivative of MVA-BN and in particular for detailed information regarding the biological assays used to determine whether a MVA virus is a derivative of MVA-BN reference is made to WO 02/42480. Thus, said term refers to vaccinia viruses showing at least one of the following features of the deposited strain MVA-BN but showing differences in one or more parts of its genome. Preferably a derivative has at least two, more preferably at least three, most preferably all of the following four features of MVA-BN:

- capability of reproductive replication in chicken embryo fibroblasts (CEF) and in the baby hamster kidney cell line BHK (ECACC 85011433), but no capability of reproductive replication in the human cell line HaCat (Boukamp *et al.* 1988, J Cell Biol. 106(3): 761-71),
- failure to replicate in vivo,
- induction of a higher immunogenicity compared to the known strain MVA 575 (ECACC V00120707) in a lethal challenge model and/or
- induction of at least substantially the same level of immunity in vaccinia virus prime/ vaccinia virus boost regimes when compared to DNA-prime/ vaccinia virus boost regimes.

In particular a derivative of MVA-BN has essentially the same replication characteristics than MVA-BN. Viruses having the same "replication characteristics" than the deposited virus are viruses that replicate with similar amplification ratios than the deposited strain in CEF cells and the cell lines BHK, HeLa, HaCat and 143B and that show a similar replication in vivo as determined in the AGR129 transgenic mouse model.

The term "not capable of reproductive replication" is used in the present application as defined in WO 02/42480. Thus, a virus that is "not capable of reproductive replication" is a virus that shows an amplification ratio of less than 1 in the human cell line HaCat (Boukamp *et al.* 1988, J Cell Biol. 106(3): 761-71. Preferably, the amplification rate of the virus used as a vector according to the invention is 0.8 or less in the human cell line HaCat. The "amplification ratio" of a virus is the ratio of virus produced from an infected cell (Output) to the amount originally used to infect the cells in the first place (Input) ("amplification ratio"). A ratio of "1" between Output and Input defines an amplification status wherein the amount of virus produced from the infected cells is the same as the amount initially used to infect the cells.

20

In the context of the definition of MVA-BN and its derivatives the term "failure to replicate in vivo" is used in the present application as defined in WO 02/42480. Thus, said term refers to viruses that do not replicate in humans and in the mice model as explained in WO 02/42480. The mice used in WO 02/42480 are incapable of producing mature B- and T-cells (AGR 129 mice). In particular MVA-BN and its derivatives do not kill AGR129 mice within a time period of at least 45 days, more preferably within at least 60 days, most preferably within 90 days after the infection of the mice with  $10^7$  pfu virus administered intra peritoneally. Preferably, the viruses that show "failure to replicate in vivo" are further characterized in that no virus can be recovered from organs or tissues of

30



the AGR129 mice 45 days, preferably 60 days and most preferably 90 days after the infection of the mice with  $10^7$  pfu virus administered intra peritoneally.

5 MVA-BN and its derivatives are preferably characterized by a higher immunogenicity compared to the known strain MVA 575 as determined in a lethal challenge mouse model as explained in WO 02/42480. In such a model unvaccinated mice die after the infection with replication competent vaccinia strains such as the Western Reserve strain L929 TK+  
10 or IHD-J. The infection with replication competent vaccinia viruses is referred to as "challenge" in the context of description of the lethal challenge model. Four days after the challenge the mice are usually killed and the viral titer in the ovaries is determined by standard plaque assays using VERO cells. The viral titer is determined for unvaccinated mice and  
15 for mice vaccinated with MVA-BN and its derivatives. More specifically MVA-BN and its derivatives are characterized in that in this test after the vaccination with  $10^2$  TCID<sub>50</sub>/ml virus the ovary virus titers are reduced by at least 70%, preferably by at least 80%, more preferably by at least 90% compared to unvaccinated mice.

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MVA-BN or its derivatives are preferably characterized by inducing at least substantially the same level of immunity in vaccinia virus prime/ vaccinia virus boost regimes when compared to DNA-prime/ vaccinia virus boost regimes. A vaccinia virus is regarded as inducing at least  
25 substantially the same level of immunity in vaccinia virus prime/ vaccinia virus boost regimes when compared to DNA-prime/ vaccinia virus boost regimes if the CTL response as measured in one of the „assay 1“ and „assay 2“ as disclosed in WO 02/42480, preferably in both assays, is at least substantially the same in vaccinia virus prime/ vaccinia virus boost regimes when compared to DNA-prime/ vaccinia virus boost regimes.  
30 More preferably the CTL response after vaccinia virus prime/vaccinia

virus boost administration is higher in at least one of the assays, when compared to DNA-prime/vaccinia virus boost regimes. Most preferably the CTL response is higher in both assays.

- 5 WO 02/42480 discloses how Vaccinia viruses are obtained having the properties of MVA-BN and its derivatives as defined above.

Methods to insert the DNA as defined above into poxviral DNA and methods to obtain recombinant poxviruses are known to the person skilled in the art. In a recombinant vaccinia virus the expression of the  
10 DNA according to the present invention is preferably, but not exclusively, under the transcriptional control of a poxvirus promoter, more preferably of a vaccinia virus promoter. The insertion of the DNA according to the present invention is preferably into a non-essential region of the virus  
15 genome. In another preferred embodiment of the invention, the heterologous nucleic acid sequence is inserted at a naturally occurring deletion site of the MVA genome (disclosed in PCT/EP96/02926).

In summary it is one of the most preferred embodiments of the present invention to provide a vector comprising the DNA as defined  
20 above, wherein the vector is MVA-BN or a derivative thereof and wherein the DNA comprises an expression cassette coding for a Flavivirus NS1 protein or part thereof, wherein the Flavivirus is preferably a Dengue virus, more preferably Dengue virus serotype 2.

25

In a preferred embodiment the invention relates to the usefulness of the NS1 protein or part thereof encoded by a DNA according to the present invention or by a vector according to the present invention for vaccination against several flaviviruses or flavivirus serotypes. For the  
30 definition of the NS1 protein or part thereof according to the present invention reference is made to the above parts of the description where

the DNA encoding NS1 has been defined by the product expressed from said DNA. The following summary regarding the protein according to the present invention is therefore not to be regarded as a limitation of the invention. In summary the NS1 protein can be an isolated NS1 protein or part thereof encoded by any Flavivirus. The NS 1 protein or part thereof is preferably derived from a Dengue virus, most preferably from Dengue virus serotype 2. The protein may only comprise the amino acid sequence of a viral NS1 protein or part thereof. In a preferred embodiment the NS1 protein may contain additional amino acids that are required for an effective expression of the protein. Examples for such amino acids/amino acid sequences are shown above and include the methionine at the N-terminus of the protein encoded by an added ATG codon and an amino acid sequence derived from the C-terminal end of the E-protein acting as a signal sequence for glycosylation of the NS1 protein or part thereof.

Other signal sequences are also within the scope of the present invention. In an alternative embodiment the NS1 amino acid sequence or a part thereof can be fused to other proteins/peptides. Examples for fusion partners are sequences allowing the identification of the protein such as tags or other flaviviral proteins or parts thereof.

20

In a preferred embodiment the present invention concerns the DNA, the vector or the NS1 protein or part thereof according to the present invention as a vaccine, in particular as a vaccine against several flaviviruses or flavivirus serotypes. A „vaccine“ is a compound, i.e. a DNA, a protein, a vector or a virus that induces a specific immune response.

25

According to one alternative of this embodiment the „vaccine“ according to the present invention is based on a Dengue virus NS1 protein or a part thereof which induces an immune response against the NS 1 proteins of all Dengue virus serotypes. In particular it has been shown that the NS1 protein of one Dengue virus serotype, in particular serotype

30

2, induces an immune response against the NS1 proteins of at least two, preferably at least three, most preferably all Dengue virus serotypes and preferably also against at least one other mosquito-borne Flavivirus..

5           As explained above the inventors of the present invention have found that the NS1 protein or part thereof according to the present invention of one Flavivirus induces an immune response against the NS1 protein of other Flaviviruses. As pointed out above the "Flavivirus" is preferably a mosquito-borne Flavivirus. In other words the inventors of the present invention have found that in an alternative embodiment the NS1  
10 protein or part thereof according to the present invention of one mosquito-borne Flavivirus induces an immune response against the NS1 protein of the mosquito-borne Flavivirus from which the vaccine is derived and also against other mosquito-borne Flaviviruses. Thus, the vaccine  
15 derived from a mosquito-borne Flavivirus is useful as vaccine against one or more mosquito-borne flaviviruses. The term "vector derived from a Flavivirus" or similar terms in the context of the present description means that a vector as defined above (e.g. a poxvirus vector or a plasmid) contains a DNA as defined above. Thus, this term refers to the vector  
20 insert and not the vector backbone. An example for a "vector derived from a Flavivirus" is a poxvirus vector, such as MVA, comprising an expression cassette comprising a poxvirus promoter, a sequence encoding a Flavivirus NS1 protein or part thereof, wherein the sequence coding for the Flavivirus NS1 protein or part thereof is preceded by an ATG codon  
25 and a sequence encoding a glycosylation signal sequence and wherein the coding sequence is terminated by a stop codon of translation.

Thus the vaccination with the DNA, the vector or the NS1 protein or part thereof is useful as a single subunit vaccine against a broad range of  
30 Flaviviruses or at least Flavivirus serotypes. The DNA or vector encoding the NS1 protein or part thereof from one Flavivirus or Flavivirus serotype

or the NS 1 protein or part thereof from said Flavivirus or serotype can thus be used as a vaccine for vaccination against other Flaviviruses and Flavivirus serotypes, respectively. For example a vaccine derived from a Dengue virus serotype 2 can be used as a vaccine against one, two or all  
5 of the serotypes 1, 3 and 4, as well as vaccine against serotype 2. It may further be useful to protect an individual against other Flaviviruses such as the West Nile Virus, the Japanese encephalitis virus and the Yellow fever virus.

10 In a preferred embodiment the DNA according to the present invention is used as a vaccine. It is known by the person skilled in the art that the administration of naked DNA harboring a eukaryotic expression cassette as in the present invention, in particular the intramuscular injection of DNA leads to the expression of the protein encoded by the  
15 expression cassette. The protein is exposed to the immune system and a specific immune response is raised.

In an alternative embodiment the vaccination is made by administering a vector according to the present invention, in particular a  
20 viral vector, more preferably a poxvirus vector, most preferably a vaccinia virus vector, e.g. a MVA vector.

For the preparation of vaccinia virus based vaccine, the virus according to the invention, in particular MVA-BN and its derivatives, is  
25 converted into a physiologically acceptable form. This can be done based on the experience in the preparation of poxvirus vaccines used for vaccination against smallpox (as described by Stickl, H. *et al.* [1974] Dtsch. med. Wschr. 99, 2386-2392). For example, the purified virus is stored at  $-80^{\circ}\text{C}$  with a titer of  $5 \times 10^8$  TCID<sub>50</sub>/ml formulated in about  
30 10mM Tris, 140 mM NaCl pH 7.4. For the preparation of vaccine shots, e.g.,  $10^2$ - $10^9$  particles of the virus are lyophilized in 100 ml of

phosphate-buffered saline (PBS) in the presence of 2% peptone and 1% human albumin in an ampoule, preferably a glass ampoule.

It is particularly preferred that the vaccinia virus based vaccine, in particular a MVA-BN based vaccine, used for vaccination is stored in a freeze-dried state. It is shown in the example section that the immune reaction as well as the percentage of cross reaction of the immune response induced by the NS1 protein of one flavivirus to the NS1 protein of different flaviviruses and flavivirus serotypes, respectively, is particularly high if the virus used for vaccination was stored as freeze dried virus. Thus, the vaccine shots preferably can be produced by stepwise freeze-drying of the virus in a formulation. This formulation can contain additional additives such as mannitol, dextran, sugar, glycine, lactose or polyvinylpyrrolidone or other additives such as antioxidants or inert gas, stabilizers or recombinant proteins (e.g. human serum albumin) suitable for *in vivo* administration. An typical virus containing formulation suitable for freeze-drying comprises 10 mM Tris-buffer, 140 mM NaCl, 18.9 g/l Dextran (MW 36000 - 40000), 45 g/l Sucrose, 0.108 g/l L-glutamic acid mono potassium salt monohydrate pH 7.4. After freeze-drying the glass ampoule is then sealed and can be stored between 4°C and room temperature for several months. However, as long as no need exists the ampoule is stored preferably at temperatures below -20°C. For vaccination the lyophilisate can be dissolved in 0.1 to 0.5 ml of an aqueous solution, such a water, physiological saline or Tris buffer, and administered either systemically or locally, i.e. by parenterally, intramuscularly or any other path of administration know to the skilled practitioner. The mode of administration, the dose and the number of administrations can be optimized by those skilled in the art in a known manner. Most preferred for poxvirus vectors is subcutaneous or intramuscular administration. Most preferably the vaccination is done by administration of two vaccine shots in an interval of e.g. 3 to 5 weeks.

If the vaccine is a MVA-BN vector or derivative thereof comprising a DNA according to the present invention a particular embodiment of the present invention concerns a kit for vaccination comprising a MVA-BN virus vector according to the present invention for the first vaccination („priming“) in a first vial/container and for a second vaccination („boosting“) in a second vial/container.

If the vaccine is a MVA-BN vector or derivative thereof comprising a DNA as defined above a particular embodiment of the present invention concerns the administration of the vaccine in therapeutically effective amounts in a first inoculation („priming inoculation“) and in a second inoculation („boosting inoculation“). The interval between the priming inoculation and the boosting inoculation is e.g. 2 to 12 weeks, preferably e.g. 3-6 weeks, more preferably e.g. about 3 weeks. The virus amount used for vaccination should be at least  $1 \times 10^2$  TCID<sub>50</sub>, preferably e.g.  $1 \times 10^7$  TCID<sub>50</sub> to  $1 \times 10^9$  TCID<sub>50</sub>. Moreover, a particular embodiment of the present invention concerns a kit for vaccination comprising a MVA-BN virus vector as defined above for the first vaccination („priming“) in a first vial/container and for a second vaccination („boosting“) in a second vial/container.

Thus, the invention concerns in the vaccine embodiments a vaccine comprising a DNA, a vector or a NS1 protein or part thereof as defined above and the use of said DNA, vector or protein for the preparation of a vaccine. According to a preferred embodiment the invention concerns the use of said DNA, vector or protein for the preparation of a vaccine wherein the NS1 protein or part thereof, the NS1 protein or part thereof encoded by the DNA or the vector is from one Dengue virus serotype and wherein the DNA, the vector or the NS 1 protein or part thereof is used as a

vaccine against two, three or all Dengue virus serotypes. Most preferably the Dengue virus serotype is serotype 2.

The invention further relates to a method for the treatment or  
5 prevention of a Flavivirus infection comprising inoculating an animal,  
including a human, in need thereof with a DNA as above, a vector as  
above or a NS1 protein or part thereof as above. In particular the  
invention relates to a method as above, wherein the NS1 protein or part  
thereof or the NS1 protein or part thereof encoded by the DNA or the  
10 vector is from one Dengue virus serotype and wherein the DNA, the vector  
or the NS 1 protein or part thereof is used as a vaccine against two, three  
or all Dengue virus serotypes



## Summary of the Invention

The invention relates in particular to the following, alone or in combination:

5

Use of

- a nucleic acid comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes at least for the NS1 protein or a part thereof of a mosquito-born flavivirus,
  - 10 - a vector comprising said nucleic acid and/or
  - a NS1 protein or part thereof of said flavivirus
- for the preparation of a vaccine against the mosquito-borne Flavivirus from which the nucleic acid or the NS 1 protein or part thereof is derived and against at least one other mosquito-borne Flavivirus.

15

Use as above, wherein the mosquito-born Flavivirus from which the nucleic acid or the NS1 protein or part thereof is derived is a Dengue virus.

20 Use of a

- nucleic acid comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes at least for the NS1 protein or a part thereof of a Dengue virus serotype,
  - vector comprising said nucleic acid and/or
  - 25 - NS1 protein or part thereof of said Dengue virus serotype
- for the preparation of a vaccine against all Dengue virus serotypes and optionally against at least one other mosquito-borne Flavivirus.

Use as above, wherein the Dengue virus from which the nucleic acid or  
30 the NS1 protein or part thereof is derived is Dengue virus serotype 2.

Use as above, wherein the sequence coding for the NS1 protein or part thereof of the mosquito-borne flavivirus or of the Dengue virus serotype is preceded by an ATG codon and a sequence encoding a glycosylation signal sequence and wherein the coding sequence is terminated by a stop  
5 codon of translation.

Use as above, wherein the other mosquito-borne Flavivirus is selected from the West Nile virus, the Yellow fever virus and the Japanese Encephalitis virus.

10

Use as above, wherein the vector is a poxvirus vector.

Use as above, wherein the poxvirus vector is a Modified Vaccinia Virus Ankara (MVA) strain, in particular MVA-BN deposited at the European  
15 Collection of Cell Cultures under number V00083008 or a derivative thereof.

Use as above, wherein the poxvirus vector is freeze-dried and is reconstituted in a pharmaceutically acceptable diluent prior to  
20 administration.

Use as above, wherein the transcriptional regulatory element is a poxvirus promoter.

25 Use as above, wherein the vaccine is administered in therapeutically effective amounts in a first inoculation ("priming inoculation") and in a second inoculation ("boosting inoculation")

Method for the treatment or prevention of a flavivirus infections  
30 comprising inoculating an animal, including a human, in need thereof with

- a nucleic acid comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes at least for the NS1 protein or a part thereof of a mosquito-borne flavivirus,
  - a vector comprising said nucleic acid and/or
  - 5 - a NS1 protein or part of said flavivirus,
- wherein the Flavivirus infection is an infection by the mosquito-borne Flavivirus from which the nucleic acid, or the NS 1 protein or part thereof is derived and/or an infection by another mosquito-borne Flavivirus.

- 10 Method as above, wherein the mosquito-borne Flavivirus from which the nucleic acid or NS1 protein or part thereof is derived is a Dengue virus.

Method for the treatment or prevention of a Flavivirus infection comprising inoculating an animal, including a human, in need thereof with

- 15 - a nucleic acid comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes at least for the NS1 protein or a part thereof of a Dengue virus serotype,
- a vector comprising said nucleic acid and/or
  - a NS1 protein or part thereof of said Dengue virus serotype,
- 20 wherein the Flavivirus infection is an infection by the Dengue virus serotype from which the nucleic acid, or the NS 1 protein or part thereof is derived and/or an infection by another Dengue virus serotypes and/or an infection by other mosquito-borne Flaviviruses.

- 25 Method as above, wherein the Dengue virus from which the DNA or the protein or part thereof is derived is Dengue virus serotype 2.

Method as above, wherein the sequence coding for the NS1 protein or part thereof of the mosquito-borne flavivirus or of the Dengue virus

30 serotype is preceded by an ATG codon and a sequence encoding a

glycosylation signal sequence and wherein the coding sequence is terminated by a stop codon of translation.

Method as above, wherein the vector is a poxvirus vector.

5

Method as above, wherein the poxvirus vector is a Modified Vaccinia Virus Ankara (MVA) strain

10

Method as above, wherein the MVA strain is MVA-BN deposited at the European Collection of Cell Cultures under number V00083008 or a derivative thereof.

15

Method as above, wherein the poxvirus vector is freeze-dried and is reconstituted in a pharmaceutically acceptable diluent prior to administration.

Method as above, wherein the transcriptional regulatory element is a poxvirus promoter.

20

Method as above, wherein the poxvirus vector or the pharmaceutical composition is administered in therapeutically effective amounts in a first inoculation ("priming inoculation") and in a second inoculation ("boosting inoculation")

25

Poxvirus vector harboring a DNA comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes at least for a Flavivirus NS1 protein or a part thereof, wherein the poxvirus is Modified Vaccinia Virus Ankara (MVA) strain BN deposited at the European Collection of Cell Cultures under number V00083008 or a derivative thereof.

30

Poxvirus vector as above, wherein the Flavivirus is a mosquito-borne Flavivirus, in particular a Dengue virus.

5 Poxvirus vector as above, wherein the Dengue virus is Dengue virus serotype 2.

Poxvirus vector as above, wherein the sequence coding for the Flavivirus NS1 protein or part thereof is preceded by an ATG codon and a sequence encoding a glycosylation signal sequence and wherein the coding  
10 sequence is terminated by a stop codon of translation.

Poxvirus vector as above, wherein the transcriptional regulatory element is a poxvirus promoter.

15 Poxvirus vector as above, wherein the poxvirus vector is freeze-dried.

Poxvirus vector as above as a vaccine.

Pharmaceutical composition comprising a poxvirus vector as above and a  
20 pharmaceutically acceptable carrier, diluent and/or additive.

Poxvirus vector as above or pharmaceutical composition as above for the treatment and/or prevention of a flavivirus infection, wherein the poxvirus vector or the pharmaceutical composition is administered in  
25 therapeutically effective amounts in a first inoculation ("priming inoculation") and in a second inoculation ("boosting inoculation")

Method for the treatment or prevention of a flavivirus infection comprising inoculating an animal, including a human, in need thereof with a vector as  
30 above or with the pharmaceutical composition as above.

Cell, preferably a human cell, comprising a poxvirus vector as above.

Use of the poxvirus vector as above for the preparation of a vaccine to treat or to prevent a Flavivirus infection.

5

Kit for prime/boost immunization comprising a poxvirus vector as above or a pharmaceutical composition as above for a first inoculation ("priming inoculation") in a first vial/container and for a second inoculation ("boosting inoculation") in a second vial/container.

10

### Short Description of the Figures

**Figure 1A:** The dengue NGC strain "signal sequence+NS1" cDNA protein coding sequence of the construct used as an example in this invention.

5 The start of the NS1 gene in the natural context is indicated by an arrow. Important features are the addition of an ATG start codon and a stop codon (in this example "TAG"). Nucleotide sequence numbers refer to position in the NGC strain genome (Genbank accession number AF038403). The nucleotide and amino acid sequence in Figure 1A  
10 corresponds to SEQ:ID No. 5. The amino acid is separately shown as SEQ:ID No. 6.

**Figure 1B:** Diagram of plasmid pAF7NS1 containing the dengue NGC strain "signal sequence+NS1" protein coding sequence.

15

**Figure 1C:** Nucleotide sequence of the NS1 cassette within plasmid pAF7 showing the primer binding sites for PCR amplification of this cassette with oBN345 and oBN338. The nucleotide and amino acid sequence in Figure 1C corresponds to SEQ:ID No. 7. The amino acid is separately  
20 shown as SEQ:ID No. 8.

**Figure 1D:** top: Kyte-Doolittle hydrophobicity plot of dengue NGC strain NS1 amino acid sequence (amino acid 776 to 1127 of dengue NCG polyprotein Genbank Accession AF038403). Values above zero = hydrophobic.

25 bottom: Kyte-Doolittle hydrophobicity plot of dengue NGC strain NS1 amino acid sequence containing a signal sequence derived from the last 28 amino acids of C-terminal of E protein (amino acid 748 to 775). The total amino acid sequence represents amino acid 748 to 1127 of the dengue NCG polyprotein (Genbank Accession AF038403) which for this strain  
30 starts with an "ATG" start codon but lacks a stop codon. Sig = Signal sequence. Values above zero = hydrophobic

**Figure 2:** Nucleotide sequence of the "poxvirus promoter+signal sequence+NS1" expression cassette. The nucleotide and amino acid sequence in Figure 2 corresponds to SEQ:ID No. 9. The amino acid is  
 5 separately shown as SEQ:ID No. 10. Briefly, the minimal poxvirus early/late promoter element controls the expression of the NS1 protein of Dengue virus serotype 2, wherein the N-terminus of the NS1 protein is fused to the 28 C-terminal aminoacids of the E-protein. The translation is terminated at an TAG stop codon that has been inserted into the nucleic  
 10 acid sequence.

**Figure 3A:** Cloning of NS1 expression cassette into the blunt ended Xho I site (blunt end cloning) of pBNX07 to produce the clone pBN41. PPr = poxvirus promoter, D2F1 = flank 1 of deletion site 2, NPT II = neomycin  
 15 resistance gene, IRES = Internal Ribosome Binding Site, EGFP = Enhanced Green Fluorescence Protein, NS1 (in pBN41) = signal sequence+NS1, D2F2 = Flank 2 of deletion site 2, Sig = signal sequence. AmpR = Ampicillin resistance gene.

**Figure 3B:** Hind III map of MVA (Genbank U94848) showing the location of the six deletion sites of MVA (-J- = junction of the deletion site). The "PPr+NPT II+IRES+EGFP+PPr+NS1" cassette was inserted into deletion 2  
 20 site of MVA. PPr = poxvirus promoter, NPT II = neomycin resistance gene (protein coding sequence), IRES = Internal Ribosome Binding Site and  
 25 NS1 = signal sequence plus NS1 protein coding sequence of dengue 2 NGC strain.

**Figure 4:** Plot of the ELISA absorbance readings of the post-immunized sera titrations for all three rabbits.



**Figure 5:** Elisa cross reactivity studies. The cross reactivity of a rabbit serum of day 38 (upper part) and day 66 (lower part) with lysates of cells infected with DENV-1, DENV-3, DENV-4, JEV and WNV was tested in an ELISA-assay.

## Examples

The following examples will further illustrate the present invention. It will be well understood by a person skilled in the art that the examples  
5 may not be interpreted in a way that limits the applicability of the technology provided by the present invention to these examples.

### Example 1: Construction of mBN07

#### 10 1. Details of NS1 antigen (Figure 1)

The example refers to NS1 of serotype 2 derived from the New Guinea C strain - NGC strain (example: Genbank sequence AF038403). Since the NS1 protein of the Flaviviruses is produced as part of a  
15 polyprotein precursor the NS1 gene in the corresponding DNA is not preceded by a „ATG“ start codon.

Therefore, a cDNA sequence coding for the NS1 protein must require the addition of a "ATG" start codon. This is then followed by the  
20 addition of a signal sequence so that the newly synthesized NS1 protein becomes glycosylated in the endoplasmic reticulum. Finally, the protein-coding cassette needs a stop codon and in this example TAG was added to the 3' terminal end of the protein coding cDNA sequence. In the example used in this invention the "ATG+signal sequence" element was  
25 derived from the hydrophobic C-terminal end of the E protein (the last 28 amino acids, which for NGC strains starts with the amino acid M (ATG)).

Fig 1A shows the exact signal sequence plus NS1 sequence used as the example for this invention (see also SEQ:ID 5 and 6). The "signal sequence+NS1" nucleotide coding sequence was obtained by RT-PCR  
30 amplification from dengue NGC genomic RNA using the following primers:

**D2NS1-1up** : 5'-ACAAGATCTGGAATGAATTACGTCACCTCA-3' (SEQ:ID No. 4)

In italics: Bgl II restriction endonuclease recognition site.

Underlined is the start codon.

5

**D2NS1-2down**: 5'-AATAGATCTCTACTAGGCTGTGACCAAGGAGTT-3' (SEQ:ID No. 3)

In italics: Bgl II restriction endonuclease recognition site.

Underlined is the stop codon.

10

The RT-PCR amplification was carried out using the Titan One Tube RT-PCR kit from Roche Molecular Biochemical (Catalog number 1-939-823) following the instructions recommended by the manufacturer.

However, essentially any commercial or non-commercial RT-PCR kit can be used instead.

15

The RT-PCR product can then be cloned into the BamHI site of any multiple cloning site present in many of the commercial bacterial cloning plasmids available but in this example it was cloned into pAF7 to give rise to clone to pAF7D2NS1 - see fig 1B and 1C for sequence details for pAF7D2NS1. Fig 1D shows the hydrophobicity plots of NS1 amino acid sequence and NS1 containing the added signal sequence from the C-terminal amino acid coding sequence of E protein. The short N-terminal hydrophobic domain is indicative of a signal sequence.

20

25

## 2. Details of NS1 expression cassette (Figure 2)

To express this "signal sequence+NS1" from a poxvirus vector such as canarypox, fowlpox, vaccinia or MVA, a poxvirus promoter needs to be added to the 5' end of this cDNA. Poly adenylation signal sequences are not required as all poxvirus synthesized RNAs are polyadenylated by a

30

virally encoded enzyme that requires no polyA addition signal sequence for carrying out this function. Any poxvirus promoter can be used for the expression of this cassette. Figure 2 and SEQ:ID No. 9 and 10 show the nucleotide sequence of the "poxvirus promoter+signal sequence+NS1" cassette used as the example in this invention.

For the example used in this invention the "signal sequence+NS1" was further PCR amplified from the NS1 plasmid clone using the primers oBN338 and oBN345. oBN345 primer contains a nucleotide sequence of a poxvirus minimal promoter element 5' to the target sequence within the cloning plasmid. Plasmid target sequence for oBN345 primer binding was approximately 40 nucleotides upstream of the signal sequence start codon. This was to ensure the RNA transcript contain a stretch of non-protein coding sequence before the signal sequence ATG start codon.

PCR primers with Ps promoter:

**oBN338:** 5'-TTGTTAGCAGCCGGATCGTAGACTTAATTA (30mer) (SEQ:ID No. 1)

**oBN345:**

5'-CAAAAAATTGAAATTTATTTTTTTTTTTTGGAATATAAATAAAA  
ACACGATAATACCATGG -3'  
(SEQ:ID No. 2)

(Underlined nucleotides present the poxvirus minimal promoter sequence.)

Annealing temperature for the PCR amplification reaction for the first five cycles was calculated from the nucleotide sequence that binds to the homologous sequence in the cloning vector of oBN345.

### 3. Integration of NS1 expression cassette into MVA (Fig 3)

The PCR amplification product was blunt end cloned into the cut and blunt ended Xho I site of plasmid pBNX07 (see Fig 3a) to form  
5 plasmid pBN41 (see Fig 3a). pBN41 is the vector for integrating the "pox promoter+signal sequence+NS1" cassette into deletion site 2 of MVA by homologous recombination.

The essential features of pBN41 (see Fig3a) are as follows:

10

- Plasmid backbone is pBluescript SK-plus from Stratagene (Genbank VB0078)
- D2F1: Deletion 2 flank 1 homologous recombination arm. This represents the nucleotide sequence from 20117 to 20717 of the MVA  
15 Genbank sequence U94848.
- PPr: Poxvirus promoter.
- NPT II: Neomycin phosphotransferase protein coding sequence (protein coding sequence of Genbank V00618).
- IRES: Internal Ribosome Entry Sequence from encephalomyocarditis  
20 virus (Jang et al., 1989, Genbank M16802).
- EGFP: Enhanced Green Fluorescence Protein coding sequence (protein coding sequence - nucleotide 675 to nucleotide 1394 of Genbank sequence U57609)
- NS1: "signal sequence+NS1" protein coding sequence from dengue  
25 NGC strain.
- D2F2: Deletion 2 Flank 2 homologous recombination arm. This represents the nucleotide sequence from 20719 to 21343 of the MVA Genbank sequence U94848.
- AmpR: Ampicillin resistance gene of pBluescript

### 3.1 Insertion of dengue "pox promoter+signal sequence+NS1" into deletion site of MVA by homologous recombination

#### *3.1.1 Integration into MVA Genome by Homologous Recombination*

5       The above integration vector pBN41 is used to integrate the dengue NS1 expression cassette plus also the reporter cassette (Pox promoter+NPT II-IRES-EGFP) into the MVA genome by homologous recombination between flank 1 and flank 2 arms of pBN41 and the homologous target sequences within the MVA genome. This is achieved  
10 by transfecting the linearized integration vector into chicken embryo fibroblast (CEF) cells previously infected with MVA at low multiplicity of infection (MOI, for example, 0.01 infectious units per cell). At 48 hours post infection or when the infection had reached confluency a viral extract is prepared and stored at -20°C ready for selection and clone purification  
15 of desired recombinant MVA (rMVA).

#### *3.1.2 Selection of rMVA and Clone Purification*

      The elimination of non-recombinant MVA (empty vector virus) and the amplification of rMVA is achieved by infection of confluent chicken  
20 embryo fibroblast (CEF) cells at low MOI in the presence of G418 (amount of G418 has to be optimize to determine the highest dose that dose not kill the CEF cells). Any virus that does not contain and integrated NPT II gene will not replicate in the presence of G418 added to the cell maintenance medium. G418 inhibits DNA replication but since the CEF  
25 cells will be in the stationary non-replicating state they will not be affected by the action of G418. CEF cells infected with rMVAs can be visualized under a fluorescence microscope due to the expression of the enhanced fluorescent green protein.

30   Viral extracts from the homologous recombination step must be serially diluted and used to infect fresh CEF cells in the presence of G418 and

overlaid with low-melting point agarose. After 2 days of infection, the agarose-infected plates are observed under a fluorescent microscope for single foci of green infected cells. These cells are marked and agarose plugs containing the infected foci of cells are taken and placed into 1.5 ml  
5 microcentrifugation tubes containing sterile cell maintenance medium. Virus is released from the agarose plug by freeze-thawing the tube three times at  $-20^{\circ}\text{C}$ .

The best clone or clones are further clone purified under agarose  
10 until by PCR analysis there is no signs of empty vector contamination (3 to 30 rounds of clone purification). These clones are then amplified for further stringent testing for correct insertion configuration, sequence verification of promoter-foreign gene cassette and expression analysis by RT-PCR. After these analysis only one clone was further amplified under  
15 G418 selection to prepare a master stock for further characterization and immunogenicity studies.

The recombinant MVA with the inserted dengue NS1 expression cassette described in this invention was named mBN07. Fig3b shows the  
20 configuration of inserted foreign sequence in mBN07.

#### **4. Expression of authentic NS1 by MVA**

25 The expression of the NS1 protein from the recombinant MVA, mBN07, was verified by standard western blot analysis under non-denaturing conditions. More particularly, NS1 expression was analyzed after purified mBN07 was used to infect mammalian tissue culture cells, for example BHK-21 cells, at an MOI of 1.0 infectious unit per cell. Crude  
30 protein extracts were prepared from these infected cells 24-30 hours after infection where portions of these extract were mixed with SDS-PAGE gel

loading buffer containing 2-mercaptoethanol (2-ME) or not containing 2-ME. These samples plus protein extract from cells (mosquito cell line) infected with dengue NGC strain as a positive control were electrophoretically separated in a SDS-PAGE gel and then blotted onto  
5 nitrocellulose membrane. The membrane was probed with an anti-dengue NS1 monoclonal antibody.

It was shown that NS1 expressed by mBN07 is recognized by the anti-dengue NS1 monoclonal antibody and forms the correct dimeric form  
10 similar to NS1 from dengue infected cells (compare unboiled mBN07 without 2-ME lane with unboiled DEN2 without 2-ME lane). Moreover it was shown that the dimeric form resolves out to the monomeric forms under denaturing conditions (see boiled mBN07 with 2-ME lane).

15 The NS1 expressed in cells infected with mBN07 was also recognized by pooled convalescent patients' sera and with monoclonal antibodies that cross-react to NS1 of all four serotypes of dengue in western blot analysis. This demonstrates that NS1 expressed by mBN07 is immunogenic.

20

Throughout the example section mBN07 (sometimes also termed BN07) is either stored in a liquid state (optionally frozen) or in a freeze-dried state. To obtain a freeze-dried virus a virus containing solution is prepared that comprises 10 mM Tris-buffer, 140 mM NaCl, 18.9 g/l  
25 Dextran (MW 36000 - 40000), 45 g/l sucrose, 0.108 g/l L-glutamic acid mono potassium salt monohydrate pH 7.4. Said formulation is then freeze-dried. For reconstitution water is added to the freeze-dried preparation.



**Example 2: Cross immunogenicity of NS1 expressed by mBN07 to NS1 of Dengue viruses other than serotype 2 and to the NS1 of Japanese encephalitis virus (JEV) and West Nile virus (WNV)**

5     **1. NS1 expressed from mBN07: reactivity to patient convalescent sera**

Tested was the possibility that NS1 expressed by mBN07 is recognized by convalescent patient sera from individuals who have evidence of previous dengue virus infection. Serum from 68 individuals  
10    who have antibodies against dengue virus envelope protein (by immunoblotting against authentic antigens prepared from Dengue serotypes 1 to 4) were selected for testing against immunoblot strips prepared from mBN07 infected cell extracts and MVA-GFP infected cell extracts as control. The antigen-containing cell lysates were treated with  
15    sample buffer without 2 mercaptoethanol and were not heated. Of the 68 individual sera tested, 62 (91.2%) reacted with BN07 NS1 expressed by mBN07 in immunoblots. These sera were further analyzed for reactivity to NS1 of all 4 dengue virus serotypes as well as Japanese encephalitis virus (JEV). The results are shown in Table 1. Fifty-four of the sera reacted with  
20    NS1 of all dengue virus serotypes and Japanese Encephalitis Virus (JEV), and 53 of these 54 sera (98.2%) also reacted with NS1 expressed from mBN07. Seven (7) sera were specific for NS1 of at least one dengue virus serotype and did not react with NS1 of JEV. All these 7 sera also reacted with NS1 expressed by mBN07. Another 7 sera reacted only with NS1 of  
25    JEV and not NS1 of any dengue virus serotype, yet 2 (28.6%) of these JEV-specific sera also reacted with NS1 expressed by mBN07.

**Table 1:**

	BN07 NS1 NEGATIVE	BN07 NS1 POSITIVE

AUTHENTIC DEN NS1 POSITIVE	0% (0/7)	100% (7/7)
AUTHENTIC DEN & JEV NS1 POSITIVE	1.85% (1/54)	98.15% (53/54)
AUTHENTIC JEV NS1 POSITIVE	71.43% (5/7)	28.57% (2/7)

Comparison of antiserum reactions against authentic NS1 and NS1 expressed by mBN07. In brackets: Number of samples tested positive/Total number of samples tested. DEN = dengue, JEV = Japanese encephalitis virus.

5

The same 68 sera were also analyzed by reactivity against premembrane proteins. In the experience of the inventors, antibodies against premembrane are far more specific than antibodies against NS1 or E. Thus patients who have been infected with dengue will produce antibodies which recognize dengue virus premembrane and not JEV premembrane and vice versa. An analysis along these lines will provide a better prediction of the history of infection of individuals. Table 2 shows that sera from 22 patients reacted with authentic dengue premembrane protein alone thus suggesting that these 22 patients have been exposed to dengue virus only and not to JEV. All these 22 sera reacted with NS1 expressed by mBN07. Another 22 patients had evidence of previous infection with both dengue and JEV, and again all 22 sera also reacted with NS1 expressed by mBN07. In this series there were also 21 patients who had evidence of previous infection with JEV only (even though these sera had cross-reactive antibodies against dengue E). Interestingly, 17 of the 21 (82%) JEV responders reacted with NS1 expressed by mBN07. There were only 3 sera in the whole set that did not react with premembrane protein of either dengue or JEV and only 1 of these reacted with NS1 expressed by mBN07. The most likely reason for this is that the antibody titer is too low to be detected by immunoblotting.

**Table 2:**

	BN07 NS1 NEGATIVE	BN07 NS1 POSITIVE
AUTHENTIC DEN prM POSITIVE	0% (0/22)	100% (22/22)
AUTHENTIC DEN & JEV prM POSITIVE	0% (0/22)	100% (22/22)
AUTHENTIC JEV prM POSITIVE	19.0% (4/21)	81.0% (17/21)
PrM NEGATIVE	66.7% (2/3)	33.3% (1/3)

Comparison of antiserum reactions against authentic premembrane and BN07 NS1. In brackets: Number of samples tested positive/Total number of samples tested. DEN = dengue, JEV = Japanese encephalitis virus.

The data in table 2 also clearly shows that of the 6 sera which did not react with NS1 expressed by mBN07, 4 were from individuals who had been previously infected with JEV and not dengue. The remaining 2 had no detectable antibodies to premembrane protein of either dengue or JEV and were likely to have been of a low titer.

## **2. mBN07 vaccination of Rabbits and testing of post-immunization sera against dengue virus and Japanese encephalitis virus immunoblots and ELISA assays**

Three specific pathogen-free rabbits were immunized by subcutaneous route according to the vaccination schedule as shown below. Each rabbit was vaccinated with one vial of freeze-dried vaccine (1x10<sup>8</sup> TCID<sub>50</sub> BN07 freeze-dried vaccine) reconstituted to 1ml with sterile water on day 0 and then again on day 28. Blood samples were taken prior to first vaccination (prebleed) and again 10 days after second vaccination.

Day 0 = prebleed followed by 1<sup>st</sup> vaccination

- Day 28 = 2<sup>nd</sup> vaccination  
Day 38 = blood sampling  
Day 56 = 3<sup>rd</sup> vaccination  
Day 66 = blood sampling  
5 Day 112 = 50ml blood withdrawal from each rabbit

## 2.1 Testing of prebleed and post-immunization sera against dengue serotype 2 immunoblots

- 10 Dengue 2 virus antigens and antigens of uninfected C6/36 cells were separated by SDS PAGE under non-denaturing conditions. For immunoblot assays serum of day 38 (diluted 1:200) was used.  
The results clearly demonstrated that upon vaccination with mBN07 all three rabbit produced anti-NS1 antibodies of high titres that cross react  
15 with authentic NS1 produced from a dengue serotype 2 infection of tissue culture mosquito cells. Serum taken before vaccination did not react to any of the dengue protein on the immunoblots.

- Post-immunized serum of day 38 was titrated at 1:1000, 1:2000,  
20 1:4000, 1:10<sup>-4</sup>, 1:10<sup>-5</sup>, 1:10<sup>-6</sup> and tested on immunoblot strips of Dengue 2 virus antigens and control strips of uninfected C6/36 cells separated by SDS PAGE under non-denaturing conditions. Endpoint titers for the three rabbit sera of day 38 was calculated to be 1:10 000. The endpoint titers for the sera of day 66 were calculated to be 1 x 10<sup>5</sup> in both the  
25 immunoblot assay and the ELISA, respectively (data not shown).

- Both, pre- and post-immunized serum were titrated at 1:10<sup>-2</sup>, 1:10<sup>-3</sup>, 1:10<sup>-4</sup>, 1:10<sup>-5</sup>, 1:10<sup>-6</sup>, 1:10<sup>-7</sup> and tested in indirect IgG ELISA. The wells were coated with dengue 2 and uninfected C6/36 lysates at 1:250  
30 dilution.

**Table 3:**

		$1:10^{-2}$	$1:10^{-3}$	$1:10^{-4}$	$1:10^{-5}$	$1:10^{-6}$	$1:10^{-7}$
Rabbit #1	Pre	-0.012	0.006	0.007	0.003	-0.002	0.001
	Post	0.623	0.127	0.02	0.004	0.001	-0.003
Rabbit #2	Pre	-0.012	0	-0.001	-0.003	0	-0.001
	Post	0.402	0.06	-0.007	0.008	-0.003	0.002
Rabbit #3	Pre	-0.008	0.03	-0.002	0.005	-0.002	-0.002
	Post	0.907	0.224	0.038	0.011	-0.001	-0.003

ELISA absorbance reading for pre- and post-immunized serum from each rabbit at different dilutions (Pre = pre-immune sera, Post = post immunization sera).

The titration results for post-immunized sera of each rabbit were plotted as shown Fig 4. The estimated endpoint titers for each rabbit post-immunized serum are 1:1000.

## 2.2 Testing of prebleed and post-immunization sera against dengue serotype 1, 3, and 4, Japanese encephalitis virus and West Nile virus immunoblots

Each of the rabbit serum of day 38 was tested at 1:1000 dilution on immunoblot strips of Dengue 1, 2, 3, 4 and JE virus antigens plus control strips of uninfected C6/36 cells separated by SDS PAGE under non-denaturing conditions. It was shown that each rabbit post-immunization sera reacts with NS1 from dengue serotypes 1, 3 and 4 as well as with NS1 on Japanese encephalitis immunoblots.

Each of the rabbit serum of day 66 was tested at 1:1000 dilution on immunoblot strips of Dengue 1, 3, 4, WNV and JE virus antigens plus control strips of uninfected C6/36 cells separated by SDS PAGE under non-denaturing conditions. It was shown that each rabbit post-

immunization sera reacts with NS1 from dengue serotypes 1, 3 and 4 as well as with NS1 on Japanese encephalitis virus and West Nile virus immunoblots.

5           To confirm the immunoblot assays Elisa cross reactivity assays were performed. The wells of microtiterplates were coated with DENV-1, DENV-3, DENV-4, JEV, WNV and uninfected cell lysates at 1:250 dilutions. The sera were sera from day 38 (Fig. 5A) and day 66 (Fig. 5B).

10           From the immunoblot assays as well as from the ELISA experiments it can be concluded that antibodies elicited by Dengue virus NS1 are cross reactive with all other Dengue serotypes, the JEV and the WNV.

### 2.3. Conclusions

15

- Rabbits immunized with mBN07 vaccine elicited antibodies that recognize authentic Dengue virus serotype 2 NS1.
- Very high immune response was observed where end points were  $1:10^4$  and  $1:10^3$  in both immunoblot assays and ELISA respectively.
- 20 - Antibodies elicited in the rabbits cross-reacted with all the other dengue serotypes (1, 3 & 4).
- The antibodies also cross-reacted with NS1 from a heterologous virus such as JEV and WNV.

### 25   3. Immunogenicity studies in mice

Female out-bred mice were immunized by the intraperitoneal route with mBN07 expressing Dengue virus NS1 in different amounts and schedules as shown below. mBN08, a MVA corresponding to mBN07 but not expressing NS1 and PBS served as control. The serum of the mice was  
30   used to check whether the antibodies generated in the mice were able to react on Western blots with NS1 proteins from the different flavivirus

serotypes and from the Japanese encephalitis virus, respectively. The sera from the control mice were negative in all experiments.

The following groups were analyzed:

5

Group	No. of mice	1 <sup>ST</sup> dose	Interval	2 <sup>ND</sup> dose	Interval before bleeding
1	9	1x10 <sup>7</sup> TCID <sub>50</sub> BN07 each (0.1ml of vaccine diluted 1:5 in PBS/mouse)	4 weeks	1x10 <sup>7</sup> TCID <sub>50</sub> BN07 each (0.1ml of vaccine diluted 1:5 in PBS/mouse)	3 weeks
2	9	1x10 <sup>7</sup> TCID <sub>50</sub> BN07 freeze-dried vaccine each (reconstituted in 1.2ml water per mouse)	4 weeks	1x10 <sup>7</sup> TCID <sub>50</sub> BN07 freeze-dried vaccine each (reconstituted in 1.2ml water per mouse)	3 weeks
3	10	1x10 <sup>7</sup> TCID <sub>50</sub> BN07 freeze-dried vaccine each (reconstituted in 1.2ml water per mouse)	3 weeks	1x10 <sup>7</sup> TCID <sub>50</sub> BN07 freeze-dried vaccine each (reconstituted in 1.2ml water per mouse)	4 weeks
4	10	1x10 <sup>7</sup> TCID <sub>50</sub> BN07 each (0.1ml of vaccine diluted 1:5 in PBS/mouse)	4 weeks	1x10 <sup>7</sup> TCID <sub>50</sub> BN07 each (0.1ml of vaccine diluted 1:5 in PBS/mouse)	4 weeks
5	10	1x10 <sup>7</sup> TCID <sub>50</sub> BN07 freeze-dried vaccine each (reconstituted in 1.2ml water per mouse)	4 weeks	1x10 <sup>7</sup> TCID <sub>50</sub> BN07 freeze-dried vaccine each (reconstituted in 1.2ml water per mouse)	4 weeks

In the immunoblot experiments the following results were obtained:

GROUP	NO. OF MICE TESTED	DENV-1 POSITIVE (% POS)	DENV-2 POSITIVE (% POS)	DENV-3 POSITIVE (% POS)	DENV-4 POSITIVE (% POS)	JEV POSITIVE (% POS)
1	9	5 (55.5%)	9 (100%)	3 (33.3%)	7 (77.7%)	5 (55.5%)
2	9	8 (88.8%)	9 (100%)	8 (88.8%)	9 (100%)	5 (55.5%)
3	10	9 (90%)	10 (100%)	9 (90%)	10 (100%)	8 (80%)
4	10	8 (80%)	10 (100%)	9 (90%)	9 (90%)	4 (40%)
5	10	10 (100%)	10 (100%)	9 (100%)	10 (100%)	8 (80%)

Very similar experiments have been obtained with Balb/c mice: Mice were vaccinated with two shots of  $1 \times 10^8$  TCID<sub>50</sub> BN07 (freeze-dried and reconstituted with water) at days 0 and 21. The sera were obtained at day 42. 100% of the sera reacted with Dengue virus 2 NS1, 100 % of the sera reacted with NS1 proteins from all four Dengue virus serotypes and 75 of the sera reacted with the NS 1 protein of JEV. The results obtained with  $1 \times 10^8$  TCID<sub>50</sub> non-freeze dried BN07 were as follows: 100% of the sera reacted with NS1 from all four Denguevirus serotypes. The sera recognized NS1 from JEV not as good as the sera obtained from mice vaccinated with freeze-dried BN07.

#### Conclusion:

15

- 100% of the mice immunized with BN07 had antibodies to DENV-2 NS1 with very strong response.
- The immune response of mice immunized with  $1 \times 10^7$  TCID<sub>50</sub> BN07 was as strong as the immune response of mice immunized with  $1 \times 10^8$  TCID<sub>50</sub> BN07 (data not shown)

20



- The best cross-reactivity percentage were observed at with mice immunized at 4 weeks interval and 4 weeks before bleeding .
- Mice immunized with the BN07 freeze-dried vaccine were observed to have much stronger response to NS1 compared to those immunized with the non-freeze dried vaccine.

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- 15 **Flamand M**, Megret F, Mathieu M, LePault, Rey FA and Deubel V., 1999. Dengue Virus Type 1 Nonstructural Glycoprotein NS1 is Secreted from mammalian cells as a soluble hexamer in a glycsylation-dependent fashion. J. Virol., 73:6104-6110.
- 20 **Jang SK**, Davies MV, Kaufman RJ and Wimmer E., 1989. Initiation of protein synthesis by internal entry of ribosomes into the 5' nontranslated region of encephalomyocarditis virus RNA in vivo. J. Virol., 63:1651-60.
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- 25 Pages 991-1041.
- Schlesinger JJ**, Brandriss MW and Walsh EE., 1987. Protection of mice against dengue 2 virus encephalitis by immunization with the dengue 2 virus non-structural protein NS1. J. Gen. Virol., 68:853-7

**Schesinger JJ**, Foltzer M and Chapman S., 1993. The Fc portion of antibody to yellow fever virus NS1 is a determinant of protection against yellow fever encephalitis in mice. *Virology*. 192: 132-41

- 5 The techniques and procedures described in this description are familiar to a skilled practitioner(s) in the art of molecular biology and virology especially relating to Flavivirus virology and genetic manipulation of poxviruses. The techniques and procedures described can be found in detail in the following literature resources:

- 10 **Molecular Cloning**, A laboratory Manual. Second Edition. By J. Sambrook, E.F. Fritsch and T. Maniatis. Cold Spring Harbor Laboratory Press. 1989.
- Virology Methods Manual**. Edited by Brian WJ Mahy and Hillar O Kangro. Academic Press. 1996.

15

**Molecular Virology: A Practical Approach**. Edited by AJ Davison and RM Elliott. The Practical Approach Series. IRL Press at Oxford University Press. Oxford 1993. Chapter 9 : Expression of genes by vaccinia virus vectors.

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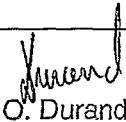
**Current Protocols in Molecular Biology**. Publisher: John Wiley and Son Inc. 1998. Chapter 16, section IV: Expression of proteins in mammalian cells using vaccinia viral vector.

- 25 **Antibodies, A Laboratory Manual**. By Ed Harlow and David Lane. Cold Spring Harbor Laboratory Press. 1988.

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

<b>A.</b> The indications made below relate to the microorganism referred to in the description on page <u>13</u> , line <u>25</u>	
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Name of depositary institution <b>ECACC</b> European Collection of Cell Cultures	
Address of depositary institution <i>(including postal code and country)</i> Centre for Applied Microbiology & Research Salisbury Wiltshire SP4 OJG, United Kingdom	
Date of deposit August 30, 2000	Accession Number 00083008
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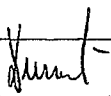
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## Claims

### 1. Use of

(i) a nucleic acid comprising an expression cassette  
comprising a transcriptional regulatory element and a  
sequence which codes at least for the NS1 protein or a  
part thereof of a mosquito-born flavivirus,

(ii) a vector comprising said nucleic acid and/or

(iii) a NS1 protein or part thereof of said flavivirus

for the preparation of a vaccine against the mosquito-borne Flavivirus  
from which the nucleic acid or the NS 1 protein or part thereof is  
derived and against at least one other mosquito-borne Flavivirus.

### 2. Use according to claim 1, wherein the mosquito-born Flavivirus

from which the nucleic acid or the NS1 protein or part thereof is  
derived is a Dengue virus.

### 3. Use of a

(i) nucleic acid comprising an expression cassette  
comprising a transcriptional regulatory element and a  
sequence which codes at least for the NS1 protein or a  
part thereof of a Dengue virus serotype,

(ii) vector comprising said nucleic acid and/or

(iii) NS1 protein or part thereof of said Dengue virus serotype

for the preparation of a vaccine against all Dengue virus serotypes and  
optionally against at least one other mosquito-borne Flavivirus.

### 4. Use according to anyone of claims 2 to 3, wherein the Dengue virus

from which the nucleic acid or the NS1 protein or part thereof is  
derived is Dengue virus serotype 2.

5. Use according to anyone of claims 1 to 4, wherein the sequence coding for the NS1 protein or part thereof of the mosquito-borne flavivirus or of the Dengue virus serotype is preceded by an ATG codon and a sequence encoding a glycosylation signal sequence and wherein the coding sequence is terminated by a stop codon of translation.  
5
6. Use according to anyone of claims 1 to 5, wherein the other mosquito-borne Flavivirus is selected from the West Nile virus, the Yellow fever virus and the Japanese encephalitis virus.  
10
7. Use according to anyone of claims 1 to 6, wherein the vector is a poxvirus vector.
8. Use according to claim 7, wherein the poxvirus vector is a Modified Vaccinia Virus Ankara (MVA) strain, in particular MVA-BN deposited at the European Collection of Cell Cultures under number V00083008 or a derivative thereof.  
15
9. Use according to anyone of claims 7 to 8, wherein the poxvirus vector is freeze-dried and is reconstituted in a pharmaceutically acceptable diluent prior to administration.  
20
10. Use according to anyone of claims 1 to 9, wherein the transcriptional regulatory element is a poxvirus promoter.  
25
11. Use according to any one of claims 1 to 10, wherein the vaccine is administered in therapeutically effective amounts in a first inoculation ("priming inoculation") and in a second inoculation ("boosting inoculation")  
30

12. Method for the treatment or prevention of a flavivirus infection comprising inoculating an animal, including a human, in need thereof with

- (i) a nucleic acid comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes at least for the NS1 protein or a part thereof of a mosquito-borne flavivirus,
- (ii) a vector comprising said nucleic acid and/or
- (iii) a NS1 protein or part thereof of said flavivirus,

wherein the Flavivirus infection is an infection by the mosquito-borne Flavivirus from which the nucleic acid, or the NS 1 protein or part thereof is derived and/or an infection by another mosquito-borne Flavivirus.

13. Method according to claim 12, wherein the mosquito-borne Flavivirus from which the nucleic acid or NS1 protein or part thereof is derived is a Dengue virus.

14. Method for the treatment or prevention of a Flavivirus infection comprising inoculating an animal, including a human, in need thereof with

- (i) a nucleic acid comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes at least for the NS1 protein or a part thereof of a Dengue virus serotype,
- (ii) a vector comprising said nucleic acid and/or
- (iii) a NS1 protein or part thereof of said Dengue virus,

wherein the Flavivirus infection is an infection by the Dengue virus serotype from which the nucleic acid, or the NS 1 protein or part thereof is derived and/or an infection by another Dengue virus serotypes and/or an infection by other mosquito-borne Flaviviruses.

15. Method according to claim 12 to 14, wherein the Dengue virus from which the DNA or the protein or part thereof is derived is Dengue virus serotype 2.
- 5
16. Method according to anyone of claims 12 to 15, wherein the sequence coding for the NS1 protein or part thereof of the mosquito-borne flavivirus or of the Dengue virus serotype is preceded by an ATG codon and a sequence encoding a glycosylation signal sequence and wherein the coding sequence is terminated by a stop codon of translation.
- 10
17. Method according to anyone of claims 12 to 16, wherein the vector is a poxvirus vector.
- 15
18. Method according to claim 17, wherein the poxvirus vector is a Modified Vaccinia Virus Ankara (MVA) strain
19. Method according to claim 18, wherein the MVA strain is MVA-BN deposited at the European Collection of Cell Cultures under number V00083008 or a derivative thereof.
- 20
20. Method according to anyone of claims 17 to 19, wherein the poxvirus vector is freeze-dried and is reconstituted in a pharmaceutically acceptable diluent prior to administration.
- 25
21. Method according to anyone of claims 12 to 20, wherein the transcriptional regulatory element is a poxvirus promoter.
22. Method according to anyone of claims 17 to 21, wherein the poxvirus vector or the pharmaceutical composition is administered
- 30



in therapeutically effective amounts in a first inoculation ("priming inoculation") and in a second inoculation ("boosting inoculation")

- 5 23. Poxvirus vector harboring a DNA comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes at least for a Flavivirus NS1 protein or a part thereof, wherein the poxvirus is Modified Vaccinia Virus Ankara (MVA) strain BN deposited at the European Collection of Cell Cultures under number V00083008 or a derivative thereof.
- 10 24. Poxvirus vector according to claim 23, wherein the Flavivirus is a mosquito-borne Flavivirus, in particular a Dengue virus.
- 15 25. Poxvirus vector according to claim 24, wherein the Dengue virus is Dengue virus serotype 2.
- 20 26. Poxvirus vector according to anyone of claims 23 to 25, wherein the sequence coding for the Flavivirus NS1 protein or part thereof is preceded by an ATG codon and a sequence encoding a glycosylation signal sequence and wherein the coding sequence is terminated by a stop codon of translation.
- 25 27. Poxvirus vector according to anyone of claims 23 to 26, wherein the transcriptional regulatory element is a poxvirus promoter.
28. Poxvirus vector according to anyone of claims 23 to 27, wherein the poxvirus vector is freeze-dried.
- 30 29. Poxvirus vector according to anyone of claims 23 to 28 as a vaccine.

30. Pharmaceutical composition comprising a poxvirus vector according to anyone of claims 23 to 28 and a pharmaceutically acceptable carrier, diluent and/or additive.

5

31. Poxvirus vector according to anyone of claims 23 to 29 or pharmaceutical composition according to claim 30 for the treatment and/or prevention of a flavivirus infection, wherein the poxvirus vector or the pharmaceutical composition is administered in therapeutically effective amounts in a first inoculation ("priming inoculation") and in a second inoculation ("boosting inoculation")

10

32. Method for the treatment or prevention of a flavivirus infection comprising inoculating an animal, including a human, in need thereof with a vector according to anyone of claims 23 to 29 or with the pharmaceutical composition according to claim 30.

15

33. Cell, preferably a human cell, comprising a poxvirus vector according to anyone of claims 23 to 28.

20

34. Use of the poxvirus vector according to anyone of claims 23 to 28 for the preparation of a vaccine to treat or to prevent a Flavivirus infection.

25

35. Kit for prime/boost immunization comprising a poxvirus vector according to anyone of claims 23 to 29 or a pharmaceutical composition according to 30 for a first inoculation ("priming inoculation") in a first vial/container and for a second inoculation ("boosting inoculation") in a second vial/container.

30

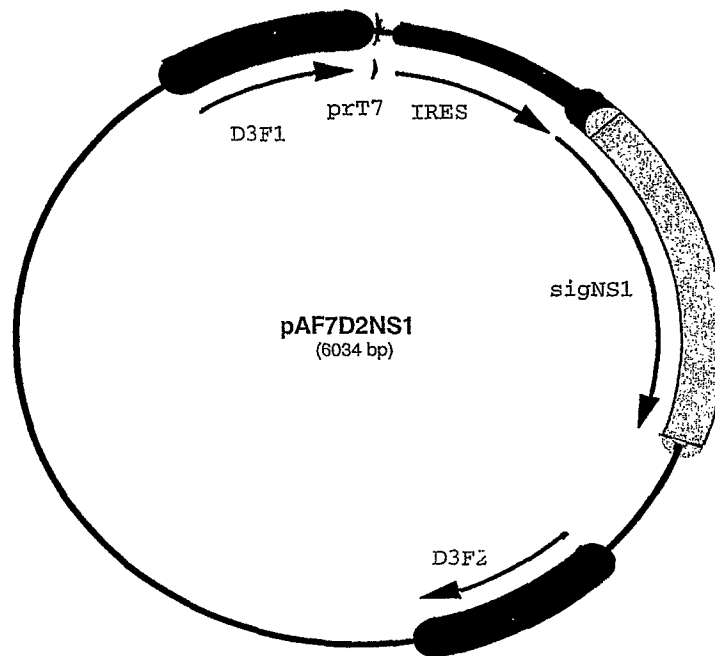
1/9

Fig. 1A:

2338 atgaattcagcagcaccctcactgtctgtgtcactagtagtattggtgggagtcgtgacgctgtatttggggagttatggtgcaggccgtagtgggtgctgtgtgagctggaa  
 1 M N S R S T S L S V S L V L V G V V T L Y L G V M V Q A D S G C V V S W K  
 Signal sequence derived from E protein  
 2448 AAACAAAGACTGAAGTGGCAGTGGGAGTTTCATCAGACAGCAACGTCACACATGGACAGAAACAATACAAGTTCCCAACCAAGATCCCCCTTCAAAAGCTAGCTTCAGCTA  
 37 N K E L K C G S G I F I T D N V H T W T E Q Y K F Q P E S P S K L A S A  
 NS1 protein coding sequence with stop codon (TAG)  
 2558 TCCAGAAAAGCTCATGAAAGAGGGCATTGTGGAAATCCGCTCAGTAACACAGACTGGAAAAATCTGATGTGGAAACAATAACACAGAAATGAATCAATTCATTCATCAAGAAAT  
 74 I Q K A H E E G I C G I R S V T R L E N L M W K Q I T P E L N H I L S E N  
 2668 GAGGTGAAGTGAAGTATATGACAGAGACATCAAGGAATCATGCGCAGGAAAAACGATCTCTGCGAGCCGCCACCTGAGCTGAAGTATTCATGTGAAAAACATGGGG  
 111 E V K L T I M T G D I K G I M Q A G K R S L Q P Q P T E L K Y S W K T W G  
 2778 CAAGCGAAATGCTCTCTACAGAGTCTCATACCAAGACCTTCTCTCATTTGATGGCCCCGAAACACAGCAGATGCCCCCAACACAAACAGAGCTTGGAAATTCGCTGGAAAGTTG  
 147 K A K M L S T E S H N Q T F L I D G P E T A E C P N T N R A W N S L E V  
 2888 AAGACTATGGCTTTGGAGTATTCACCAACCAATATATAGCTAAAGTTGAGAGAAAGCAGAGATGTATTCTGCGACTCAAAAACCTCATGTCTCAGCGGCCCATAAAAGACACACAG  
 184 E D Y G F G V F T N I W L K L R E K Q D V F C D S K L M S A A I K D N R  
 2998 GCCCTCCATGCCGATATGGGTATTGGATAGAAAGTGCACTCAATGACACATGGAAGATAGAGAAAGCCCTCTTCTCGAAGTTAAAAGCTGCCACTGCCCAAGTCA  
 221 A V H A D M G Y W I E S A L N D T W K I E K A S F I E V K S C H W P K S H  
 3108 CACCCTCTGGAGTAATGGAGTTAGAAAGTGAGATGATAATCCAAAGAAATTCGCTGGACCAAGTGTCAACACAACTACAGACCGGCTACCATACACAAACAGCAG  
 257 T L W S N G V L E S E M I I P K N F A G P V S Q H N Y R P G Y H T Q T A  
 3218 GACCATGGCATCTAGGTAAAGCTTGAGATGGACTTTGATTCTCGAAGGAACCAACAGTGGTGGTGAATAGAGGACCCCTCTTTAAGAAACAACACTACT  
 294 G P W H L G K L E M D F D F C E G T T V V T E D C G N R G P S L R T T T  
 3328 GCCTCTGGAACCACTATAACAGAAATGGTGTCTGCCGATCTTGTGCATTACCAACCGCTAAGATACAGAGGTGAGGACCGGATGCTGGTACGGGATGGAATCAGACCATTTGAA  
 331 A S G K L I T E W C C R S C T L P P L R Y R G E D G C W Y G M E I R P L K  
 3438 AGAGAAAGAAAGAAATTTGGTCAACTCTTGGTCACAGCCctag  
 367 E K E N L V N S L V T A .

2/9

Fig. 1B:



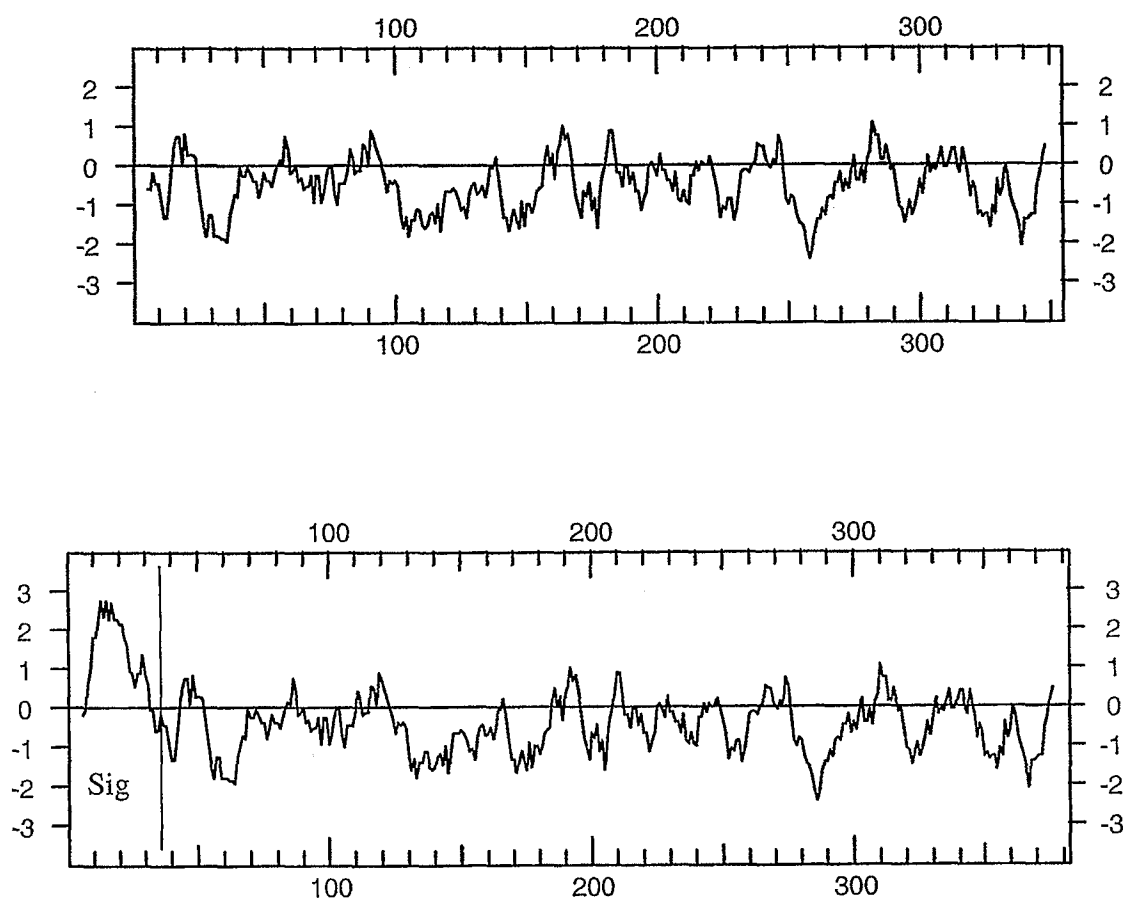
3/9

Fig. 1C

[illegible]

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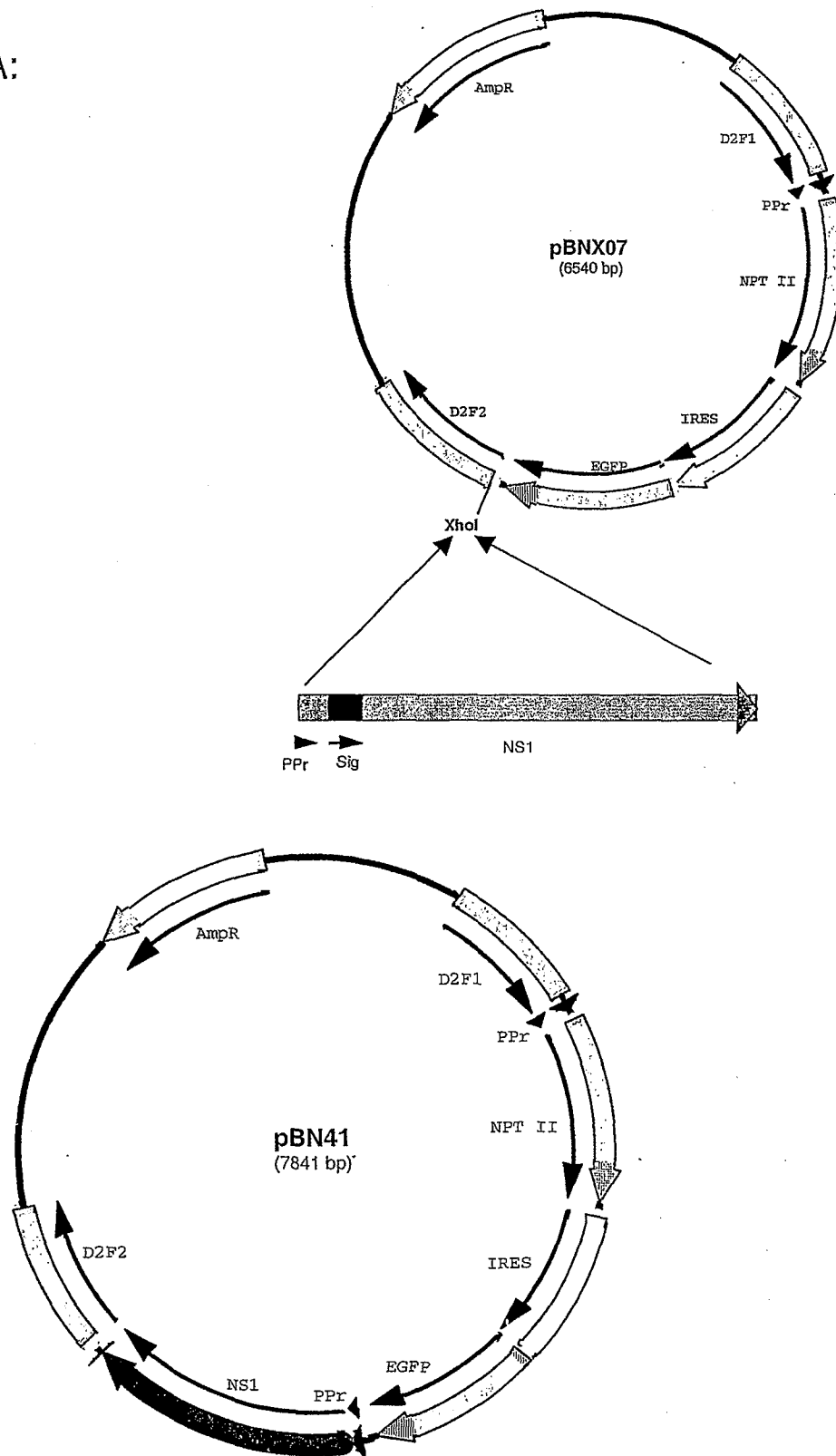
Fig. 1D:



[illegible]

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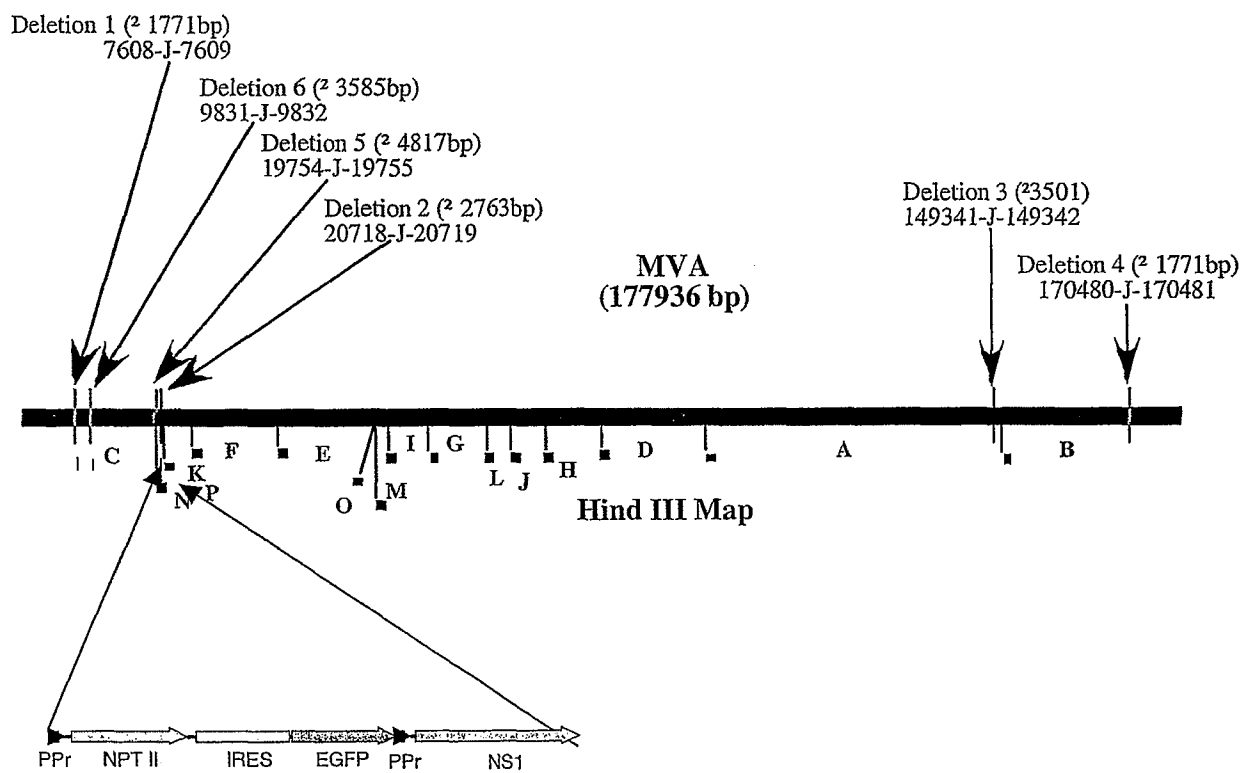
Fig. 3A:





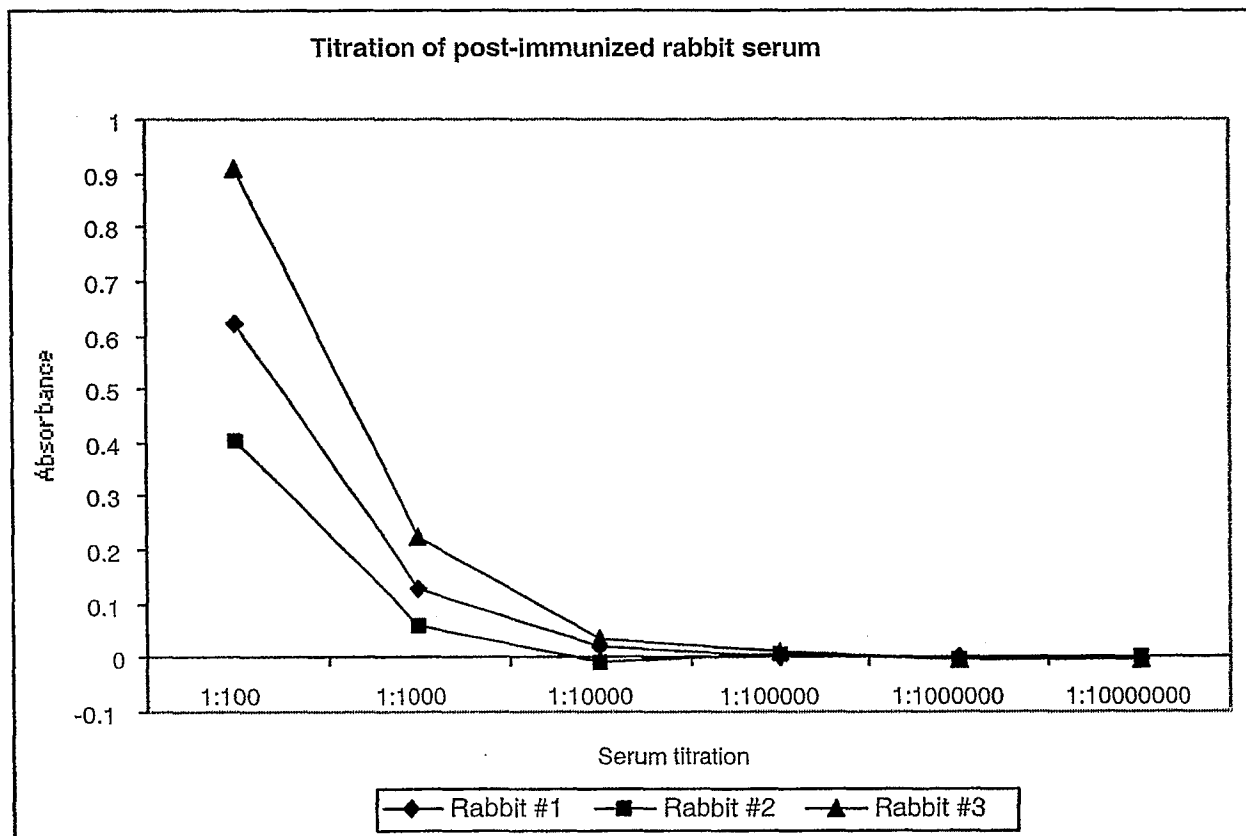
7/9

Fig. 3B:



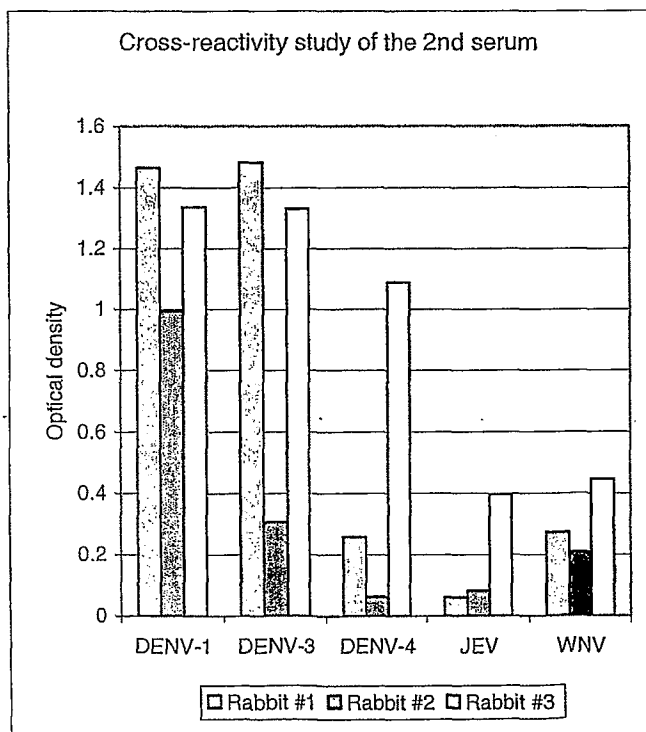
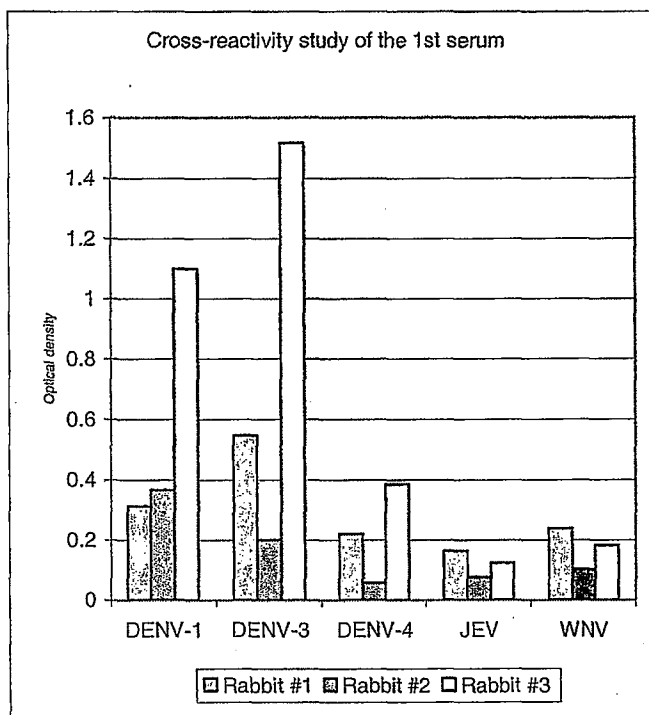
8/9

Fig. 4:



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Fig. 5:



## SEQUENCE LISTING

<110> Bavarian Nordic A/S  
Venture Technologies Sdn Bhd

<120> Flavivirus NS1 Subunit Vaccine

<130> NS1-Dengue

<140> 0000

<141> 2001-12-04

<160> 10

<170> PatentIn Ver. 2.1

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<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

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30

<210> 2

<211> 62

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<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

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gg 62

<210> 3

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

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aatagatctc tactaggctg tgaccaagga gtt

33

&lt;210&gt; 4

&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: primer

&lt;400&gt; 4

acaagatctg gaatgaattc acgtagcacc tca

33

&lt;210&gt; 5

&lt;211&gt; 1143

&lt;212&gt; DNA

&lt;213&gt; Dengue virus type 2

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(1140)

&lt;223&gt; Signal sequence of Eprotein + NS1 coding sequence

&lt;400&gt; 5

atg aat tca cgc agc acc tca ctg tct gtg tca cta gta ttg gtg gga 48

Met Asn Ser Arg Ser Thr Ser Leu Ser Val Ser Leu Val Leu Val Gly

1 5 10 15

gtc gtg acg ctg tat ttg gga gtt atg gtg cag gcc gat agt ggt tgc 96

Val Val Thr Leu Tyr Leu Gly Val Met Val Gln Ala Asp Ser Gly Cys

20 25 30

gtt gtg agc tgg aaa aac aaa gaa ctg aag tgt ggc agt ggg att ttc 144

Val Val Ser Trp Lys Asn Lys Glu Leu Lys Cys Gly Ser Gly Ile Phe

35 40 45

atc aca gac aac gtg cac aca tgg aca gaa caa tac aag ttc caa cca 192

Ile Thr Asp Asn Val His Thr Trp Thr Glu Gln Tyr Lys Phe Gln Pro

50 55 60

gaa tcc cct tca aag cta gct tca gct atc cag aaa gct cat gaa gag 240

Glu Ser Pro Ser Lys Leu Ala Ser Ala Ile Gln Lys Ala His Glu Glu

65 70 75 80

ggc att tgt gga atc cgc tca gta aca aga ctg gaa aat ctg atg tgg	288
Gly Ile Cys Gly Ile Arg Ser Val Thr Arg Leu Glu Asn Leu Met Trp	
85 90 95	
aaa caa ata aca cca gaa ttg aat cac att cta tca gaa aat gag gtg	336
Lys Gln Ile Thr Pro Glu Leu Asn His Ile Leu Ser Glu Asn Glu Val	
100 105 110	
aag ttg act att atg aca gga gac atc aaa gga atc atg cag gca gga	384
Lys Leu Thr Ile Met Thr Gly Asp Ile Lys Gly Ile Met Gln Ala Gly	
115 120 125	
aaa cga tct ctg cag ccc cag ccc act gag ctg aag tat tca tgg aaa	432
Lys Arg Ser Leu Gln Pro Gln Pro Thr Glu Leu Lys Tyr Ser Trp Lys	
130 135 140	
aca tgg ggc aaa gcg aaa atg ctc tct aca gag tct cat aac cag acc	480
Thr Trp Gly Lys Ala Lys Met Leu Ser Thr Glu Ser His Asn Gln Thr	
145 150 155 160	
ttt ctc att gat ggc ccc gaa aca gca gaa tgc ccc aac aca aac aga	528
Phe Leu Ile Asp Gly Pro Glu Thr Ala Glu Cys Pro Asn Thr Asn Arg	
165 170 175	
gct tgg aat tcg ctg gaa gtt gaa gac tat ggc ttt gga gta ttc acc	576
Ala Trp Asn Ser Leu Glu Val Glu Asp Tyr Gly Phe Gly Val Phe Thr	
180 185 190	
acc aat ata tgg cta aag ttg aga gaa aag cag gat gta ttc tgc gac	624
Thr Asn Ile Trp Leu Lys Leu Arg Glu Lys Gln Asp Val Phe Cys Asp	
195 200 205	
tca aaa ctc atg tca gcg gcc ata aaa gac aac aga gcc gtc cat gcc	672
Ser Lys Leu Met Ser Ala Ala Ile Lys Asp Asn Arg Ala Val His Ala	
210 215 220	
gat atg ggt tat tgg ata gaa agt gca ctc aat gac aca tgg aag ata	720
Asp Met Gly Tyr Trp Ile Glu Ser Ala Leu Asn Asp Thr Trp Lys Ile	
225 230 235 240	
gag aaa gcc tct ttc atc gaa gtt aaa agc tgc cac tgg cca aag tca	768
Glu Lys Ala Ser Phe Ile Glu Val Lys Ser Cys His Trp Pro Lys Ser	
245 250 255	
cac acc ctc tgg agt aat gga gtg tta gaa agt gag atg ata att cca	816
His Thr Leu Trp Ser Asn Gly Val Leu Glu Ser Glu Met Ile Ile Pro	
260 265 270	

aag aat ttc gct gga cca gtg tca caa cac aac tac aga cca ggc tac 864  
Lys Asn Phe Ala Gly Pro Val Ser Gln His Asn Tyr Arg Pro Gly Tyr  
275 280 285

cat aca caa aca gca gga cca tgg cat cta ggt aag ctt gag atg gac 912  
His Thr Gln Thr Ala Gly Pro Trp His Leu Gly Lys Leu Glu Met Asp  
290 295 300

ttt gat ttc tgc gaa gga acc aca gtg gtg gtg act gag gac tgt gga 960  
Phe Asp Phe Cys Glu Gly Thr Thr Val Val Val Thr Glu Asp Cys Gly  
305 310 315 320

aat aga gga ccc tct tta aga aca act act gcc tct gga aaa ctc ata 1008  
Asn Arg Gly Pro Ser Leu Arg Thr Thr Thr Ala Ser Gly Lys Leu Ile  
325 330 335

aca gaa tgg tgc tgc cga tct tgc aca tta cca ccg cta aga tac aga 1056  
Thr Glu Trp Cys Cys Arg Ser Cys Thr Leu Pro Pro Leu Arg Tyr Arg  
340 345 350

ggg gag gac gga tgc tgg tac ggg atg gaa atc aga cca ttg aaa gag 1104  
Gly Glu Asp Gly Cys Trp Tyr Gly Met Glu Ile Arg Pro Leu Lys Glu  
355 360 365

aaa gaa gag aat ttg gtc aac tcc ttg gtc aca gcc tag 1143  
Lys Glu Glu Asn Leu Val Asn Ser Leu Val Thr Ala  
370 375 380

<210> 6  
<211> 380  
<212> PRT  
<213> Dengue virus type 2

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1 5 10 15  
Val Val Thr Leu Tyr Leu Gly Val Met Val Gln Ala Asp Ser Gly Cys  
20 25 30  
Val Val Ser Trp Lys Asn Lys Glu Leu Lys Cys Gly Ser Gly Ile Phe  
35 40 45  
Ile Thr Asp Asn Val His Thr Trp Thr Glu Gln Tyr Lys Phe Gln Pro  
50 55 60  
Glu Ser Pro Ser Lys Leu Ala Ser Ala Ile Gln Lys Ala His Glu Glu

65	70	75	80
Gly Ile Cys Gly Ile Arg Ser Val Thr Arg Leu Glu Asn Leu Met Trp	85	90	95
Lys Gln Ile Thr Pro Glu Leu Asn His Ile Leu Ser Glu Asn Glu Val	100	105	110
Lys Leu Thr Ile Met Thr Gly Asp Ile Lys Gly Ile Met Gln Ala Gly	115	120	125
Lys Arg Ser Leu Gln Pro Gln Pro Thr Glu Leu Lys Tyr Ser Trp Lys	130	135	140
Thr Trp Gly Lys Ala Lys Met Leu Ser Thr Glu Ser His Asn Gln Thr	145	150	155
Phe Leu Ile Asp Gly Pro Glu Thr Ala Glu Cys Pro Asn Thr Asn Arg	165	170	175
Ala Trp Asn Ser Leu Glu Val Glu Asp Tyr Gly Phe Gly Val Phe Thr	180	185	190
Thr Asn Ile Trp Leu Lys Leu Arg Glu Lys Gln Asp Val Phe Cys Asp	195	200	205
Ser Lys Leu Met Ser Ala Ala Ile Lys Asp Asn Arg Ala Val His Ala	210	215	220
Asp Met Gly Tyr Trp Ile Glu Ser Ala Leu Asn Asp Thr Trp Lys Ile	225	230	235
Glu Lys Ala Ser Phe Ile Glu Val Lys Ser Cys His Trp Pro Lys Ser	245	250	255
His Thr Leu Trp Ser Asn Gly Val Leu Glu Ser Glu Met Ile Ile Pro	260	265	270
Lys Asn Phe Ala Gly Pro Val Ser Gln His Asn Tyr Arg Pro Gly Tyr	275	280	285
His Thr Gln Thr Ala Gly Pro Trp His Leu Gly Lys Leu Glu Met Asp	290	295	300
Phe Asp Phe Cys Glu Gly Thr Thr Val Val Val Thr Glu Asp Cys Gly	305	310	315
Asn Arg Gly Pro Ser Leu Arg Thr Thr Thr Ala Ser Gly Lys Leu Ile			



	325		330		335
Thr Glu Trp Cys Cys Arg Ser Cys Thr Leu Pro Pro Leu Arg Tyr Arg					
	340		345		350
Gly Glu Asp Gly Cys Trp Tyr Gly Met Glu Ile Arg Pro Leu Lys Glu					
	355		360		365
Lys Glu Glu Asn Leu Val Asn Ser Leu Val Thr Ala					
	370		375		380

&lt;210&gt; 7

&lt;211&gt; 1296

&lt;212&gt; DNA

&lt;213&gt; Dengue virus type 2

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (50) ..(1189)

&lt;220&gt;

&lt;221&gt; primer\_bind

&lt;222&gt; (12) ..(32)

&lt;220&gt;

&lt;221&gt; primer\_bind

&lt;222&gt; (1243) ..(1273)

&lt;400&gt; 7

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Met Asn Ser

1

cgc agc acc tca ctg tct gtg tca cta gta ttg gtg gga gtc gtg acg 106

Arg Ser Thr Ser Leu Ser Val Ser Leu Val Leu Val Gly Val Val Thr

5

10

15

ctg tat ttg gga gtt atg gtg cag gcc gat agt ggt tgc gtt gtg agc 154

Leu Tyr Leu Gly Val Met Val Gln Ala Asp Ser Gly Cys Val Val Ser

20

25

30

35

tgg aaa aac aaa gaa ctg aag tgt ggc agt ggg att ttc atc aca gac 202

Trp Lys Asn Lys Glu Leu Lys Cys Gly Ser Gly Ile Phe Ile Thr Asp

40

45

50

aac gtg cac aca tgg aca gaa caa tac aag ttc caa cca gaa tcc cct 250

Asn	Val	His	Thr	Trp	Thr	Glu	Gln	Tyr	Lys	Phe	Gln	Pro	Glu	Ser	Pro		
			55					60					65				
tca	aag	cta	gct	tca	gct	atc	cag	aaa	gct	cat	gaa	gag	ggc	att	tgt	298	
Ser	Lys	Leu	Ala	Ser	Ala	Ile	Gln	Lys	Ala	His	Glu	Glu	Gly	Ile	Cys		
		70					75					80					
gga	atc	cgc	tca	gta	aca	aga	ctg	gaa	aat	ctg	atg	tgg	aaa	caa	ata	346	
Gly	Ile	Arg	Ser	Val	Thr	Arg	Leu	Glu	Asn	Leu	Met	Trp	Lys	Gln	Ile		
	85					90				95							
aca	cca	gaa	ttg	aat	cac	att	cta	tca	gaa	aat	gag	gtg	aag	ttg	act	394	
Thr	Pro	Glu	Leu	Asn	His	Ile	Leu	Ser	Glu	Asn	Glu	Val	Lys	Leu	Thr		
100					105				110					115			
att	atg	aca	gga	gac	atc	aaa	gga	atc	atg	cag	gca	gga	aaa	cga	tct	442	
Ile	Met	Thr	Gly	Asp	Ile	Lys	Gly	Ile	Met	Gln	Ala	Gly	Lys	Arg	Ser		
			120				125					130					
ctg	cag	ccc	cag	ccc	act	gag	ctg	aag	tat	tca	tgg	aaa	aca	tgg	ggc	490	
Leu	Gln	Pro	Gln	Pro	Thr	Glu	Leu	Lys	Tyr	Ser	Trp	Lys	Thr	Trp	Gly		
		135					140					145					
aaa	gcg	aaa	atg	ctc	tct	aca	gag	tct	cat	aac	cag	acc	ttt	ctc	att	538	
Lys	Ala	Lys	Met	Leu	Ser	Thr	Glu	Ser	His	Asn	Gln	Thr	Phe	Leu	Ile		
	150						155				160						
gat	ggc	ccc	gaa	aca	gca	gaa	tgc	ccc	aac	aca	aac	aga	gct	tgg	aat	586	
Asp	Gly	Pro	Glu	Thr	Ala	Glu	Cys	Pro	Asn	Thr	Asn	Arg	Ala	Trp	Asn		
	165					170				175							
tcg	ctg	gaa	gtt	gaa	gac	tat	ggc	ttt	gga	gta	ttc	acc	acc	aat	ata	634	
Ser	Leu	Glu	Val	Glu	Asp	Tyr	Gly	Phe	Gly	Val	Phe	Thr	Thr	Asn	Ile		
180				185				190						195			
tgg	cta	aag	ttg	aga	gaa	aag	cag	gat	gta	ttc	tgc	gac	tca	aaa	ctc	682	
Trp	Leu	Lys	Leu	Arg	Glu	Lys	Gln	Asp	Val	Phe	Cys	Asp	Ser	Lys	Leu		
			200				205					210					
atg	tca	gcg	gcc	ata	aaa	gac	aac	aga	gcc	gtc	cat	gcc	gat	atg	ggc	730	
Met	Ser	Ala	Ala	Ile	Lys	Asp	Asn	Arg	Ala	Val	His	Ala	Asp	Met	Gly		
		215					220				225						
tat	tgg	ata	gaa	agt	gca	ctc	aat	gac	aca	tgg	aag	ata	gag	aaa	gcc	778	
Tyr	Trp	Ile	Glu	Ser	Ala	Leu	Asn	Asp	Thr	Trp	Lys	Ile	Glu	Lys	Ala		
		230				235					240						
tct	ttc	atc	gaa	gtt	aaa	agc	tgc	cac	tgg	cca	aag	tca	cac	acc	ctc	826	

Ser Phe Ile Glu Val Lys Ser Cys His Trp Pro Lys Ser His Thr Leu  
 245 250 255  
  
 tgg agt aat gga gtg tta gaa agt gag atg ata att cca aag aat ttc 874  
 Trp Ser Asn Gly Val Leu Glu Ser Glu Met Ile Ile Pro Lys Asn Phe  
 260 265 270 275  
  
 gct gga cca gtg tca caa cac aac tac aga cca ggc tac cat aca caa 922  
 Ala Gly Pro Val Ser Gln His Asn Tyr Arg Pro Gly Tyr His Thr Gln  
 280 285 290  
  
 aca gca gga cca tgg cat cta ggt aag ctt gag atg gac ttt gat ttc 970  
 Thr Ala Gly Pro Trp His Leu Gly Lys Leu Glu Met Asp Phe Asp Phe  
 295 300 305  
  
 tgc gaa gga acc aca gtg gtg gtg act gag gac tgt gga aat aga gga 1018  
 Cys Glu Gly Thr Thr Val Val Val Thr Glu Asp Cys Gly Asn Arg Gly  
 310 315 320  
  
 ccc tct tta aga aca act act gcc tct gga aaa ctc ata aca gaa tgg 1066  
 Pro Ser Leu Arg Thr Thr Thr Ala Ser Gly Lys Leu Ile Thr Glu Trp  
 325 330 335  
  
 tgc tgc cga tct tgc aca tta cca ccg cta aga tac aga ggt gag gac 1114  
 Cys Cys Arg Ser Cys Thr Leu Pro Pro Leu Arg Tyr Arg Gly Glu Asp  
 340 345 350 355  
  
 gga tgc tgg tac ggg atg gaa atc aga cca ttg aaa gag aaa gaa gag 1162  
 Gly Cys Trp Tyr Gly Met Glu Ile Arg Pro Leu Lys Glu Lys Glu Glu  
 360 365 370  
  
 aat ttg gtc aac tcc ttg gtc aca gcc tagtagggat cgggggagct 1209  
 Asn Leu Val Asn Ser Leu Val Thr Ala  
 375 380  
  
 cactagtgga tccctccagc tcgagaggnc taattaatta agtctacgat ccggctgcta 1269  
  
 acaaagccccg aaaggaagct gagttgg 1296

&lt;210&gt; 8

&lt;211&gt; 380

&lt;212&gt; PRT

&lt;213&gt; Dengue virus type 2

&lt;400&gt; 8

Met Asn Ser Arg Ser Thr Ser Leu Ser Val Ser Leu Val Leu Val Gly

1

5

10

15

Val	Val	Thr	Leu	Tyr	Leu	Gly	Val	Met	Val	Gln	Ala	Asp	Ser	Gly	Cys	20	25	30
Val	Val	Ser	Trp	Lys	Asn	Lys	Glu	Leu	Lys	Cys	Gly	Ser	Gly	Ile	Phe	35	40	45
Ile	Thr	Asp	Asn	Val	His	Thr	Trp	Thr	Glu	Gln	Tyr	Lys	Phe	Gln	Pro	50	55	60
Glu	Ser	Pro	Ser	Lys	Leu	Ala	Ser	Ala	Ile	Gln	Lys	Ala	His	Glu	Glu	65	70	75
Gly	Ile	Cys	Gly	Ile	Arg	Ser	Val	Thr	Arg	Leu	Glu	Asn	Leu	Met	Trp	85	90	95
Lys	Gln	Ile	Thr	Pro	Glu	Leu	Asn	His	Ile	Leu	Ser	Glu	Asn	Glu	Val	100	105	110
Lys	Leu	Thr	Ile	Met	Thr	Gly	Asp	Ile	Lys	Gly	Ile	Met	Gln	Ala	Gly	115	120	125
Lys	Arg	Ser	Leu	Gln	Pro	Gln	Pro	Thr	Glu	Leu	Lys	Tyr	Ser	Trp	Lys	130	135	140
Thr	Trp	Gly	Lys	Ala	Lys	Met	Leu	Ser	Thr	Glu	Ser	His	Asn	Gln	Thr	145	150	155
Phe	Leu	Ile	Asp	Gly	Pro	Glu	Thr	Ala	Glu	Cys	Pro	Asn	Thr	Asn	Arg	165	170	175
Ala	Trp	Asn	Ser	Leu	Glu	Val	Glu	Asp	Tyr	Gly	Phe	Gly	Val	Phe	Thr	180	185	190
Thr	Asn	Ile	Trp	Leu	Lys	Leu	Arg	Glu	Lys	Gln	Asp	Val	Phe	Cys	Asp	195	200	205
Ser	Lys	Leu	Met	Ser	Ala	Ala	Ile	Lys	Asp	Asn	Arg	Ala	Val	His	Ala	210	215	220
Asp	Met	Gly	Tyr	Trp	Ile	Glu	Ser	Ala	Leu	Asn	Asp	Thr	Trp	Lys	Ile	225	230	235
Glu	Lys	Ala	Ser	Phe	Ile	Glu	Val	Lys	Ser	Cys	His	Trp	Pro	Lys	Ser	245	250	255
His	Thr	Leu	Trp	Ser	Asn	Gly	Val	Leu	Glu	Ser	Glu	Met	Ile	Ile	Pro	260	265	270

Lys Asn Phe Ala Gly Pro Val Ser Gln His Asn Tyr Arg Pro Gly Tyr  
 275 280 285

His Thr Gln Thr Ala Gly Pro Trp His Leu Gly Lys Leu Glu Met Asp  
 290 295 300

Phe Asp Phe Cys Glu Gly Thr Thr Val Val Val Thr Glu Asp Cys Gly  
 305 310 315 320

Asn Arg Gly Pro Ser Leu Arg Thr Thr Thr Ala Ser Gly Lys Leu Ile  
 325 330 335

Thr Glu Trp Cys Cys Arg Ser Cys Thr Leu Pro Pro Leu Arg Tyr Arg  
 340 345 350

Gly Glu Asp Gly Cys Trp Tyr Gly Met Glu Ile Arg Pro Leu Lys Glu  
 355 360 365

Lys Glu Glu Asn Leu Val Asn Ser Leu Val Thr Ala  
 370 375 380

<210> 9

<211> 1227

<212> DNA

<213> Dengue virus type 2

<220>

<221> promoter

<222> (5)..(48)

<223> minimal poxvirus promoter element

<220>

<221> CDS

<222> (85)..(1224)

<223> NS1

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accatgggaa ttccccgatc tgga atg aat tca cgc agc acc tca ctg tct 111

Met Asn Ser Arg Ser Thr Ser Leu Ser

1

5

gtg tca cta gta ttg gtg gga gtc gtg acg ctg tat ttg gga gtt atg 159

Val Ser Leu Val Leu Val Gly Val Val Thr Leu Tyr Leu Gly Val Met

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gtg cag gcc gat agt ggt tgc gtt gtg agc tgg aaa aac aaa gaa ctg				207
Val Gln Ala Asp Ser Gly Cys Val Val Ser Trp Lys Asn Lys Glu Leu				
	30	35	40	
aag tgt ggc agt ggg att ttc atc aca gac aac gtg cac aca tgg aca				255
Lys Cys Gly Ser Gly Ile Phe Ile Thr Asp Asn Val His Thr Trp Thr				
	45	50	55	
gaa caa tac aag ttc caa cca gaa tcc cct tca aag cta gct tca gct				303
Glu Gln Tyr Lys Phe Gln Pro Glu Ser Pro Ser Lys Leu Ala Ser Ala				
	60	65	70	
atc cag aaa gct cat gaa gag ggc att tgt gga atc cgc tca gta aca				351
Ile Gln Lys Ala His Glu Glu Gly Ile Cys Gly Ile Arg Ser Val Thr				
	75	80	85	
aga ctg gaa aat ctg atg tgg aaa caa ata aca cca gaa ttg aat cac				399
Arg Leu Glu Asn Leu Met Trp Lys Gln Ile Thr Pro Glu Leu Asn His				
	90	95	100	105
att cta tca gaa aat gag gtg aag ttg act att atg aca gga gac atc				447
Ile Leu Ser Glu Asn Glu Val Lys Leu Thr Ile Met Thr Gly Asp Ile				
	110	115	120	
aaa gga atc atg cag gca gga aaa cga tct ctg cag ccc cag ccc act				495
Lys Gly Ile Met Gln Ala Gly Lys Arg Ser Leu Gln Pro Gln Pro Thr				
	125	130	135	
gag ctg aag tat tca tgg aaa aca tgg ggc aaa gcg aaa atg ctc tct				543
Glu Leu Lys Tyr Ser Trp Lys Thr Trp Gly Lys Ala Lys Met Leu Ser				
	140	145	150	
aca gag tct cat aac cag acc ttt ctc att gat ggc ccc gaa aca gca				591
Thr Glu Ser His Asn Gln Thr Phe Leu Ile Asp Gly Pro Glu Thr Ala				
	155	160	165	
gaa tgc ccc aac aca aac aga gct tgg aat tcg ctg gaa gtt gaa gac				639
Glu Cys Pro Asn Thr Asn Arg Ala Trp Asn Ser Leu Glu Val Glu Asp				
	170	175	180	185
tat ggc ttt gga gta ttc acc acc aat ata tgg cta aag ttg aga gaa				687
Tyr Gly Phe Gly Val Phe Thr Thr Asn Ile Trp Leu Lys Leu Arg Glu				
	190	195	200	
aag cag gat gta ttc tgc gac tca aaa ctc atg tca gcg gcc ata aaa				735
Lys Gln Asp Val Phe Cys Asp Ser Lys Leu Met Ser Ala Ala Ile Lys				

205	210	215	
gac aac aga gcc gtc cat gcc gat atg ggt tat tgg ata gaa agt gca			783
Asp Asn Arg Ala Val His Ala Asp Met Gly Tyr Trp Ile Glu Ser Ala			
220	225	230	
ctc aat gac aca tgg aag ata gag aaa gcc tct ttc atc gaa gtt aaa			831
Leu Asn Asp Thr Trp Lys Ile Glu Lys Ala Ser Phe Ile Glu Val Lys			
235	240	245	
agc tgc cac tgg cca aag tca cac acc ctc tgg agt aat gga gtg tta			879
Ser Cys His Trp Pro Lys Ser His Thr Leu Trp Ser Asn Gly Val Leu			
250	255	260	265
gaa agt gag atg ata att cca aag aat ttc gct gga cca gtg tca caa			927
Glu Ser Glu Met Ile Ile Pro Lys Asn Phe Ala Gly Pro Val Ser Gln			
270	275	280	
cac aac tac aga cca ggc tac cat aca caa aca gca gga cca tgg cat			975
His Asn Tyr Arg Pro Gly Tyr His Thr Gln Thr Ala Gly Pro Trp His			
285	290	295	
cta ggt aag ctt gag atg gac ttt gat ttc tgc gaa gga acc aca gtg			1023
Leu Gly Lys Leu Glu Met Asp Phe Asp Phe Cys Glu Gly Thr Thr Val			
300	305	310	
gtg gtg act gag gac tgt gga aat aga gga ccc tct tta aga aca act			1071
Val Val Thr Glu Asp Cys Gly Asn Arg Gly Pro Ser Leu Arg Thr Thr			
315	320	325	
act gcc tct gga aaa ctc ata aca gaa tgg tgc tgc cga tct tgc aca			1119
Thr Ala Ser Gly Lys Leu Ile Thr Glu Trp Cys Cys Arg Ser Cys Thr			
330	335	340	345
tta cca ccg cta aga tac aga ggt gag gac gga tgc tgg tac ggg atg			1167
Leu Pro Pro Leu Arg Tyr Arg Gly Glu Asp Gly Cys Trp Tyr Gly Met			
350	355	360	
gaa atc aga cca ttg aaa gag aaa gaa gag aat ttg gtc aac tcc ttg			1215
Glu Ile Arg Pro Leu Lys Glu Lys Glu Glu Asn Leu Val Asn Ser Leu			
365	370	375	
gtc aca gcc tag			1227
Val Thr Ala			
380			

&lt;210&gt; 10

&lt;211&gt; 380

&lt;212&gt; PRT

&lt;213&gt; Dengue virus type 2

&lt;400&gt; 10

Met Asn Ser Arg Ser Thr Ser Leu Ser Val Ser Leu Val Leu Val Gly  
 1 5 10 15

Val Val Thr Leu Tyr Leu Gly Val Met Val Gln Ala Asp Ser Gly Cys  
 20 25 30

Val Val Ser Trp Lys Asn Lys Glu Leu Lys Cys Gly Ser Gly Ile Phe  
 35 40 45

Ile Thr Asp Asn Val His Thr Trp Thr Glu Gln Tyr Lys Phe Gln Pro  
 50 55 60

Glu Ser Pro Ser Lys Leu Ala Ser Ala Ile Gln Lys Ala His Glu Glu  
 65 70 75 80

Gly Ile Cys Gly Ile Arg Ser Val Thr Arg Leu Glu Asn Leu Met Trp  
 85 90 95

Lys Gln Ile Thr Pro Glu Leu Asn His Ile Leu Ser Glu Asn Glu Val  
 100 105 110

Lys Leu Thr Ile Met Thr Gly Asp Ile Lys Gly Ile Met Gln Ala Gly  
 115 120 125

Lys Arg Ser Leu Gln Pro Gln Pro Thr Glu Leu Lys Tyr Ser Trp Lys  
 130 135 140

Thr Trp Gly Lys Ala Lys Met Leu Ser Thr Glu Ser His Asn Gln Thr  
 145 150 155 160

Phe Leu Ile Asp Gly Pro Glu Thr Ala Glu Cys Pro Asn Thr Asn Arg  
 165 170 175

Ala Trp Asn Ser Leu Glu Val Glu Asp Tyr Gly Phe Gly Val Phe Thr  
 180 185 190

Thr Asn Ile Trp Leu Lys Leu Arg Glu Lys Gln Asp Val Phe Cys Asp  
 195 200 205

Ser Lys Leu Met Ser Ala Ala Ile Lys Asp Asn Arg Ala Val His Ala  
 210 215 220

Asp Met Gly Tyr Trp Ile Glu Ser Ala Leu Asn Asp Thr Trp Lys Ile



225	230	235	240
Glu Lys Ala Ser Phe Ile Glu Val Lys Ser Cys His Trp Pro Lys Ser			
245	250	255	
His Thr Leu Trp Ser Asn Gly Val Leu Glu Ser Glu Met Ile Ile Pro			
260	265	270	
Lys Asn Phe Ala Gly Pro Val Ser Gln His Asn Tyr Arg Pro Gly Tyr			
275	280	285	
His Thr Gln Thr Ala Gly Pro Trp His Leu Gly Lys Leu Glu Met Asp			
290	295	300	
Phe Asp Phe Cys Glu Gly Thr Thr Val Val Val Thr Glu Asp Cys Gly			
305	310	315	320
Asn Arg Gly Pro Ser Leu Arg Thr Thr Thr Ala Ser Gly Lys Leu Ile			
325	330	335	
Thr Glu Trp Cys Cys Arg Ser Cys Thr Leu Pro Pro Leu Arg Tyr Arg			
340	345	350	
Gly Glu Asp Gly Cys Trp Tyr Gly Met Glu Ile Arg Pro Leu Lys Glu			
355	360	365	
Lys Glu Glu Asn Leu Val Asn Ser Leu Val Thr Ala			
370	375	380	

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Organization  
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PA 2001 01804 4 December 2001 (04.12.2001) DK
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: FLAVIVIRUS NS1 SUBUNIT VACCINE

(57) Abstract: The present invention relates to NS1 proteins or parts thereof of Flaviviruses, in particular of Dengue viruses useful for vaccination against said Flavivirus and against one or more other Flaviviruses. The invention further concerns the NS1 protein or parts thereof of one Dengue virus serotype, in particular serotype 2, useful for vaccination against Dengue viruses from all serotypes. The invention further concerns DNA comprising an expression cassette coding for a Flavivirus NS1 or parts thereof, vectors, comprising said DNA and vaccines containing or expressing a Flavivirus NS1.



WO 2003/048184 A3

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C07K14/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, EMBASE, SEQUENCE SEARCH, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 60847 A (BUTRAPET SIRITORN ;BHAMARAPRAVATI NATTH (TH); KINNEY CLAIRE Y H (U) 23 August 2001 (2001-08-23) page 5, line 1 -page 8, line 25 page 11, line 6 -page 25, line 6 claims; examples	1-6, 12-16
Y	---	1-35
X	US 5 824 506 A (CHAN LILY ET AL) 20 October 1998 (1998-10-20) column 8, line 56 -column 15, line 48; claims ----- -/--	1-4,6, 12-15

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
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- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*G\* document member of the same patent family

Date of the actual completion of the international search

6 August 2003

Date of mailing of the international search report

22/08/2003

Name and mailing address of the ISA

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Sommer, B

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCHLESINGER JJ ET AL: "Protection of mice against dengue 2 virus encephalitis by immunization with the dengue 2 virus non-structural glycoprotein NS1" JOURNAL OF GENERAL VIROLOGY, vol. 68, no. 3, 1987, pages 853-858, XP009015072 ISSN: 0022-1317 abstract page 855, paragraph 2 -page 856, paragraph 3  ---	1-4, 12-15
X	CHAMBERS TJ ET AL: "Vaccine development against dengue and Japanese encephalitis: report of a World Health Organization meeting" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 15, no. 14, 1 October 1997 (1997-10-01), pages 1494-1502, XP004090331 ISSN: 0264-410X page 1499, right-hand column, paragraph 1 -page 1500, left-hand column, paragraph 1  ---	1-4, 12-15
X	BRAY M ET AL: "Monkeys immunized with intertypic chimeric dengue viruses are protected against wild-type virus challenge." JOURNAL OF VIROLOGY, vol. 70, no. 6, 1996, pages 4162-4166, XP002250234 ISSN: 0022-538X abstract  ---	1-3, 12-14
X	WO 90 01946 A (US HEALTH) 8 March 1990 (1990-03-08) page 7, line 3 -page 10, line 30  ---	23-35
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P,Y	WO 02 42480 A (MEISINGER CHRISTINE ;HOWLEY PAUL (DE); CHAPLIN PAUL (DE); BAVARIAN) 30 May 2002 (2002-05-30) page 17, paragraph 3 -page 20, paragraph 2; claims  ---  -/--	1-35

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>FALCONAR AK ET AL: "Production of dimer-specific and dengue virus group cross-reactive mouse monoclonal antibodies to the dengue 2 virus non-structural glycoprotein NS1"</p> <p>JOURNAL OF GENERAL VIROLOGY, READING, BERKS, GB, vol. 72, no. 4, April 1991 (1991-04), pages 961-965, XP000890192 ISSN: 0022-1317 abstract; table 1 page 963, right-hand column, paragraph 2 -page 965, left-hand column, paragraph 1 -----</p>	1-35

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/EP 02/12991

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claims 12-22 and 32 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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